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A Functional Analysis of the Kaposi's Sarcoma-Associated Herpesvirus G Protein-Coupled Receptor

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Abstract

Human herpesvirus-8, or KSHV, was discovered in 1994 and is the causative agent of all forms of Kaposi's sarcoma (KS). It is also associated with two lymphoproliferative disorders: primary effusion lymphoma (PEL) and multicentric Castleman's disease. The KSHV viral chemokine receptor, vGPCR, is a homologue of the IL8 receptors CXCR1 and CXCR2. vGPCR is considered a viral oncogene: it transforms fibroblasts in vitro and enhances growth and longevity of primary endothelial cells.

vGPCR signals promiscuously via several heterotrimeric G-protein subtypes and activates the MAP and SAP kinases, the Src family kinases, and PI3 kinase. However, little is known about vGPCR in the context of KSHV-infected haematopoietic cells. In this thesis, a tetracycline-inducible vGPCR-expressing PEL line is used to show that vGPCR causes a G0/G1 arrest in PEL cells via inhibition of Cdk2. This lack of Cdk2 function inhibits the chemically mediated KSHV latent-to-lytic switch. We hypothesize that expression of vGPCR outside of the normal lytic phase could inhibit virion production and resultant cell death; this would allow the many vGPCR-induced cytokines to have a more prolonged effect on the tumour microenvironment. These effects would result in enhanced angiogenesis, a hallmark of KS, as well as the recruitment of new infectable KSHV cells.

The vGPCR is among the most promising targets for rationally designed anti-KSHV therapy. However, a better understanding of vGPCR signalling events is required. This thesis also examines how vGPCR affects the function of the protein tyrosine phosphatases (PTPs), a family of enzymes that regulates many extracellular signalling events. PTPs are an exciting new potential target in anti-infective and anti-tumour therapeutics.

Lastly, we examine the inhibition of TGFβ signalling by KSHV in PEL cells. PEL cells secrete TGFβ but are resistant to its effects due to downregulation of TGFβ RII. This suggests a KSHV-mediated anti-immune strategy that requires further exploration.

Acknowledgements

After finishing my clinical training in infectious diseases at Cornell in New York City, I started as a novice in the laboratory of Ethel Cesarman. Through her guidance I grew to appreciate the complexities of molecular herpesvirology, and with her support I made my first scientific progress. When I came to UCL four years later, Professor Chris Boshoff showed me the same encouragement and support. I greatly appreciate his advice regarding not only my lab work, but also about the challenges of trying to integrate myself into a new scientific and medical community in the UK. Coming to UCL to attain a PhD while an employee of Cornell was logistically challenging; I could not have done it without the understanding and flexibility of my clinical Chief, Dr. Warren Johnson, and the Division's administrator Deborah Young. My program directors at NIH also deserve thanks for allowing me to keep my funding while I have been enrolled as a PhD student in a foreign country. It is this flexibility that young clinician-scientists need to juggle the demands of their clinical work and scientific endeavours.

My deepest gratitude goes to my wife, Nicola Philpott. Any scientific achievements I can claim would not have been possible without her moral and scientific support.

And finally, I dedicate this thesis to my father, Lawrence Cannon, who instilled in me a curiousity about science and the natural world.

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Abbreviations

AP-1	activator protein 1
APS	ammonium persulfate
ATP	adenosine triphosphate
BrdU	bromodeoxyuridine
CDI	cyclin-dependent kinase inhibitor
Cdk	cyclin delendent kinase
CMV	cytomegalovirus
CO-IP	co-immunoprecipitation
CREB	cAMP-element binding protein
DMSA	dimethyl sulphoxide
DN	dominant negative
DNA	deoxyribonucleic acid
Doxy	doxycycline
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular regulated kinase
FCS	foetal calf serum
G418	neomycin sulphate
GFX	GF 109203X
GPCR	G protein-coupled receptor
HEPES	N'-(2-hydroxyethel) piperazine N'-(2-ethanesulphonic acid)
HHV8	human herpesvirus 8
HVS	*Herpesvirus saimiri*
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
KSHV	Kaposi's sarcoma-associated herpesvirus
KS	Kaposi's sarcoma
MAPK	mitogen-activated protein kinase

β-ME	β-mercaptoethanol
MCD	multicentric Castleman's disease
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa B
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PMSF	phenyl-methyl-sulphonyl fluoride
PCR	polymerase chain reaction
PD	PD98059
PEL	primary effusion lymphoma
PTX	pertussis toxin
Rb	retinoblastoma protein
RIPA	radioimmunoprecipitation
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SH2	src-homology domains
SHP	src homology 2-domain-containing tyrosine phosphatase
STAT	signal transducer and activator of transcription
TGFβ	transforming growth factor β
Tween-20	polyoxyethylene-sorbitan monolaurate
TPA	tetradecanoyl phorbol acetate
VEGF	vascular endothelial growth factor
vGPCR	KSHV viral GPCR
Wort	wortmannin

Scientific Units

bp	base pairs
°C	degrees centigrade
g	gram
kD	Kilodalton
M	molar
m	milli
μ	micro
n	nano
rpm	revolutions per minute
v/v	volume for volume
w/v	weight for volume

Single amino acid code

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asaparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

CHAPTER 1: INTRODUCTION

1.1 Overview

Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8 (HHV-8), was discovered in 1994 when two small fragments of its genome were detected in an AIDS-related Kaposi's sarcoma (KS) lesion by representational difference analysis, a PCR-based subtractive hybridization technique (49). Since then, KSHV has been found invariably in KS lesions of all four epidemiologic types, and infection with KSHV has been shown to precede and predict the development of KS in HIV-infected patients (28, 51, 73, 281). KSHV is a γ-2-herpesvirus and the first member of the genus *Rhadinovirus* known to infect humans. Epstein-Barr virus is its closest related human pathogen but several related primate and murine viruses have also been studied (see Fig 1.1). Consistent with the lymphotropic nature of γ-herpesviruses, KSHV has also been found in lymph nodes, peripheral blood B cells, and is present in all forms of a subset of NHL called primary effusion lymphoma (PEL)(44). PEL is a rare malignancy occurring primarily, but not solely, in patients with AIDS and account for 3% to 5% of AIDS-related NHL (186). It is characterized by malignant effusions frequently without solid tumor masses, tends to occur late in HIV infection, and portends high morbidity and mortality. KSHV is also highly associated with another B-cell proliferative disorder called multicentric Castleman's disease (MCD). This systemic form of Castleman's

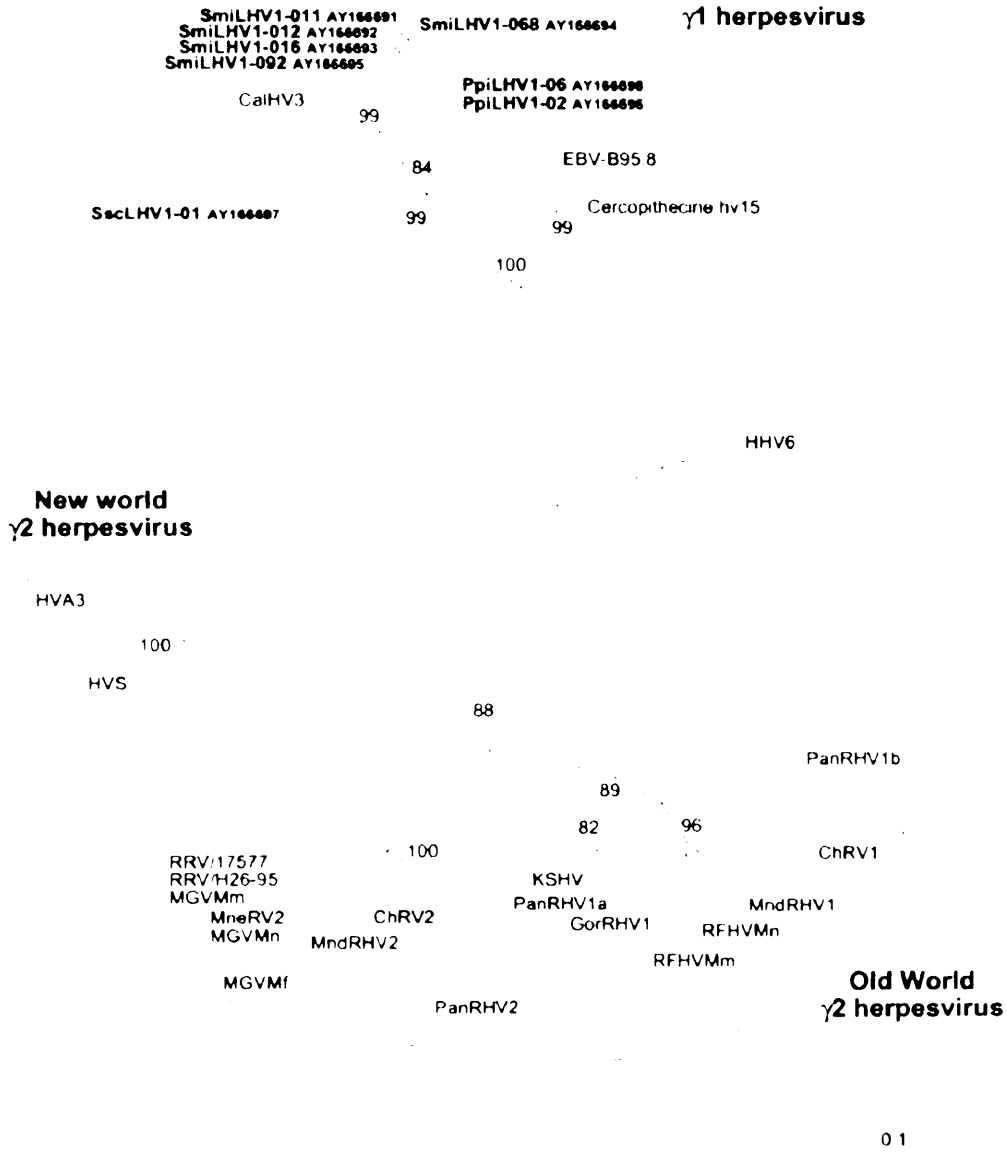

Figure 1.1 Phylogenetic tree of KSHV and related herpesviruses. Neighbor-joining protein distance tree for the 142 amino acid residues encoded by the 426-bp fragment of DNA polymerase. The branch lengths are proportional to the evolutionary distance between the taxa.
From *deThoisy et al, J Virol. 2003 Aug;77(16):9099-105*

disease presents with generalized lymphadenopathy, fever, lethargy, and immunological abnormalities. Furthermore, it has been shown that patients with MCD are at increased risk of B-cell lymphomas and KS (211).

Although KS, PEL and MCD can occur in its absence, HIV is a risk factor for all three proliferative disorders. Seroepidemiologic evidence shows that KSHV infection alone is not sufficient to cause disease, and that HIV infection or other non-HIV co-factors must be present (131). A satisfying description of the pathogenesis of KSHV-mediated disease is not yet complete but great progress has been made recently. The role of KSHV is gradually being clarified as are the similarities between HIV and non-HIV risk factors that allow KSHV infection to cause disease.

1.2 Kaposi's Sarcoma

In 1872, a Hungarian dermatologist named Moriz Kaposi reported several cases of a multifocal pigmented sarcoma in elderly Viennese men. These purplish cutaneous lesions occurred sporadically and were typically on the lower extremities. All the patients described by Kaposi eventually died of what is now known as "classic" Kaposi's sarcoma (KS). For unclear reasons, the disorder predominantly affects older men of Mediterranean and eastern European descent. Since Kaposi's initial description, three more forms of KS have been identified. The second has been known in Africa for decades. "Endemic-African" KS is much more aggressive than classic KS and typically involves lymph nodes rather than skin. It has a high mortality rate, often affects HIV-negative hosts, and targets the young (300). A

third, iatrogenic form of KS occurs within a few months of solid organ transplant in patients on immunosuppressive medications. Although most common in transplant patients on cyclosporin, it has been known to occur in those on corticosteroids. Like classic KS, this form is more common in those of Mediterranean ancestry (59, 88). A fourth and very aggressive type of KS was described in the early 1980s in otherwise healthy homosexual men (89). Along with Pneumocystis carinii pneumonia, this new form of KS heralded the onset of the AIDS epidemic and became the most common AIDS-related malignancy. AIDS-KS involves not only skin and lymph node, but often disseminates to the lung, gastrointestinal tract, liver and spleen. Early in the epidemic, lifetime incidence of KS in gay men was around 50%. The incidence started to decline in the 1980s and then more precipitously with the introduction of effective HIV therapy in the late 1990s (150).

The histogenesis of the KS spindle cell has not been easy to trace. Although KS cells stain for certain endothelial cell markers like CD34 and factor VIII, some studies show that they express proteins similar to dendritic cells, macrophages or smooth muscle cells (261). It is debated therefore whether KS cells represent a heterogeneous population of cells or instead arise from a pluripotent mesenchymal precursor cell. More recent cell surface marker studies suggest that spindle cells may be of lymphatic endothelial cell origin (129, 279).

Another area of controversy involves whether KS represents a clonal, neoplastic process or a polyclonal inflammatory lesion. In early KS lesions, the spindle cell is low in number compared to the surrounding inflammatory cells. Furthermore, KS cells in culture are dependent on exogenous growth factors and

when implanted into nude mice can induce an inflammatory and angiogenic reaction, but do not induce tumors, as would fully transformed cells (232). Moreover, regression of KS can happen spontaneously or when immunosuppression is corrected. Such characteristics along with the multifocality of KS lesions argue that the process is primarily one of dysregulated inflammation. Confusing the picture, however, X chromosome inactivation studies within single lesions as well as comparisons of multiple lesions from a single patient support a clonal origin in a subset of advanced cases (219). More recent studies have shown varying monoclonality, oligoclonality and polyclonality from lesions of various patients (102). Furthermore, three neoplastic cell lines have been established from KS lesions (20). As discussed later, one possibility is that KS starts as a hyperplastic polyclonal lesion that later gives rise to a clonal cell population only under specific circumstances and selective pressures. KS may be similar to post-transplantation lymphoproliferative disorders, which are EBV-driven B cell proliferations that may progress from a polyclonal hyperplasia, to monoclonal tumors with no evident genetic abnormalities, to frank malignant lymphomas with oncogene and tumor suppressor gene alterations (43, 140).

In 1994, Chang and colleagues used representational difference analysis (RDA) to detect two novel DNA sequences from a KS specimen that were present in much lower copy number in undiseased tissue from the same patient (49). The technique had not yet been used to find new viruses but in this case the isolated sequences showed 30-50% amino acid homology to known gamma herpesviruses including EBV. The association of KSHV with KS became stronger as multiple

investigators found KSHV sequences in virtually 100% of lesions of all four epidemiologic forms. KSHV could not, however, be propagated in culture from KS lesions and it took the establishment of PEL cell lines (see below) harboring the virus in latent form to make further seroepidemiologic studies possible (47).

1.3 Epidemiology of KSHV Infection

Of the many criteria for establishing KSHV as an etiologic agent of KS, some require an epidemiologic approach. There are numerous serologic assays to detect anti-KSHV antibodies using PEL cell lines as a source of latent or lytic antigen; the latter requiring chemical induction of viral replication. Immunofluorescence assays (IFA) to detect serum antibody to a viral latent nuclear antigen (LNA) have become the most commonly used (131). Although far from perfect, these assays are a great improvement over the previous PCR-based methods and allow more reliable comparison of various KS risk groups to each other and to control populations. Other serologic assays have included immunoblotting and ELISA using whole virus or recombinant viral antigens (159).

In the United States, the seroprevalence of KSHV in the general population is likely between 5% and 10%. In gay, HIV-positive men the seroprevalence is 30%, and in HIV-positive women and hemophiliacs, prevalence is 3-4% (94). In parts of Africa, where KS is a much more common disease and often affects HIV-negative hosts, seroprevalence may be 25-50% in the general population and appears to increase with age (201). These findings correlate well with the incidence of KS in

the same groups. Interestingly, using IFA to multiple lytic and latent KSHV antigens derived from a stimulated PEL cell line, some investigators claim a much higher rate of KSHV in several groups tested. Lennette *et al.* found 90% of American homosexual men to be seropositive as well as 25% of healthy blood donors and 2-8% of children (154). Regardless of discrepancies among different studies in seroprevalence rates, all of them indicate that KSHV infection is not ubiquitous, and that KS is not as prevalent as KSHV seropositivity. Together, these conclusions indicate that if KSHV is necessary for developing KS, it is certainly not sufficient.

Even supported by the pathologic evidence discussed above, population-based studies showing that KSHV seroprevalence and KS disease overlap do not prove a causal relationship between KSHV and KS. If KSHV infection is a necessary element in KS pathogenesis, then at some point infection must precede the full development of disease. Indeed, multiple comparisons of patients with KS, those at risk of KS, and HIV-negative control groups have shown that KSHV sequences in peripheral blood predict the development of KS (180). One well controlled study in particular used the same tissue type (i.e. peripheral blood mononuclear cells) to look for KSHV sequences in AIDS-KS patients and controls over time. KSHV could be detected prior to KS development in 52% of those who eventually manifested KS but in only 9-13% of controls (180). The presence of anti-KSHV antibody is another approach to establish prior infection. One group followed forty HIV-positive patients for up to eight years to show that KSHV seroconversion preceded KS in a high proportion of AIDS-related KS (94). Martin *et al.* studied 400 HIV-positive men and found that anti-LNA seropositivity was independently

associated with subsequent KS. Anti-LNA antibody was present at baseline in 38% of men reporting any homosexual activity and in none of the strictly heterosexual men in the control group. Seropositivity directly correlated with a personal history of sexually transmitted disease and with number of sex partners. In all, the lifetime probability of developing KS in men who were both HIV and KSHV positive was 50% (168).

1.4 KSHV Transmission

Although methods of detecting KSHV infection vary in accuracy, it is clear that groups at risk of KS always have a higher incidence of KSHV infection than their respective controls. The absolute numbers may not be perfectly accurate, but it appears that KSHV infection is not as wide spread in the general population as are some other human herpesviruses. Epidemiologic studies have argued for a sexual route of KSHV transmission within some risk groups, but unfortunately, a detailed understanding of transmission is still lacking.

As noted above, KSHV seropositivity does correlate with number of sexual partners in gay men (168). Precisely which sexual practices are riskiest is unknown. KSHV DNA has been detected in the saliva and oral tissues of seropositive patients, in the semen of AIDS-KS patients, and in the gastrointestinal mucosa from HIV-infected patients (82, 115, 269, 281). Virus shed in semen would not likely explain all transmission in either classic-Mediterranean or endemic-African

KS, but transmission via saliva may explain infection in some non-HIV groups. Indeed, the linear increase in KSHV seropositivity with age reported in Zambia by Olsen *et al* argues for a horizontal, non-sexual mode of transmission (201, 235). Other studies in Uganda and Zambia document KSHV infection in infants less than 1 year old and argue for early horizontal rather than vertical transmission (300). There has been documented KSHV transmission via organ allograft, but most iatrogenic KS patients are likely KSHV infected prior to immunosuppressive therapy (221).

1.5 Primary Effusion Lymphoma

Malignant lymphomas are the second most common HIV-associated neoplasm and having AIDS-related KS is a known risk factor (21). Most AIDS-related lymphomas are B-cell NHLs. Of these, approximately 40% are Burkitt or Burkitt-like (BL), 30% are large cell (LCL), and 30% are large cell immunoblastic lymphomas (IBL) (139). Several less common AIDS-related NHL have also been described, including cases of malignant lymphoma occurring as body cavity effusions with other distinguishing features, such as a null cell phenotype and presence of EBV (110, 140). Homology with other lymphotropic herpesviruses prompted some to study both AIDS and non-AIDS related lymphomas and led to the observation that KSHV DNA was detectable only in lymphomatous effusions (47, 49). These studies suggested that KSHV-associated lymphomas represent a distinct diagnostic entity, called body cavity-based lymphoma (BCBL) or, more recently, primary effusion lymphoma (46). This malignancy accounts for 3% to 5% of AIDS-related NHL and has a unique set of

morphological, immunophenotypic and molecular genetic characteristics that distinguish it from other AIDS-related NHL.

Clinically, PEL is an aggressive lymphoma that grows in liquid phase and has a predilection for spreading along serous membranes without an infiltrative or destructive growth pattern. Spreading throughout pleura, pericardium and peritoneum can sometimes be so extensive as to make the site of origin undeterminable at autopsy. These malignant effusions occur largely without associated solid tumor or mass, hence the term 'primary 'effusion lymphoma as distinct from the common effusions complicating many types of NHL. However, approximately 15% of KSHV-associated lymphomas in HIV-positive patients present as solid extranodal tumors, with about half of these developing a subsequent lymphomatous effusion. Histologically, the most common appearance of PEL is a large mixture of immunoblast-like cells along with other anaplastic, multilobulated or multinucleated cells resembling the Reed-Sternberg cells of Hodgkin's disease. Numerous mitotic figures are present and there is evident necrosis of some tumor cells with subsequent lysis and disintegration to form granular background material (186).

In addition to KSHV infection, there are other interesting genetic features of PEL. Co-infection with EBV is common and usually clonal, but interactions between EBV and KSHV are not yet clear. PEL tumors lack c-myc gene rearrangements and most also lack bcl-2, ras, and p53 alterations (45). While the vast majority of tumors have a B-cell genotype, tumor cells usually do not express B- and T-cell associated antigens, although they do display various markers of activation such as

CD30, CD38, and CD71 (11). PEL-derived cells in culture retain the KSHV genome as a large nuclear episome at 40-80 copies per cell (47). Typically, these cells are of clonal origin and are transformed by standard criteria (91, 231). KSHV gene expression in PEL cell lines is consistent with latent infection in which lytic genes necessary for viral replication are expressed in only a small proportion of cells (84).

The epidemiology of PEL correlates with the distribution of KSHV infection. Most cases have been found in men, frequently HIV-infected with homosexuality as a risk factor. Similar to KS, the median age of PEL onset is younger in AIDS-related cases.

1.6 Multicentric Castleman's Disease

At this writing, the only other AIDS-related disease that is convincingly associated with KSHV infection is multicentric Castleman's disease (MCD) (203). Although Castleman's disease (CD) was originally described as a localized lymphoid hyperplasia, MCD was later described as a systemic variant (90). AIDS patients are particularly prone to this systemic form and although there is histologic overlap, MCD is more often of the plasma cell type than the hyaline-vascular type of CD usually seen in localized disease. MCD is found more often in men than women and incidence increases with age. Patients develop multiple lymphadenopathies and a variety of constitutional symptoms. Cytopenias, rashes and intercurrent infections are not unusual. Interestingly, some patients with MCD have been reported to develop NHL. Additionally, the plasma cell histologic type of MCD is a risk factor for KS (253).

The combination of pathologic evidence and epidemiologic associations with other KSHV-related disease argues a strong case for KSHV as an etiologic agent in MCD. Soulier *et al* looked for KSHV DNA sequences in samples from 31 Castleman's disease patients and found evidence of infection in all 14 of those with concomitant HIV infection but in only 7 of the 17 HIV-negative subjects (253). Further work showed that KSHV is found almost universally in HIV-associated MCD specimens but in only 40% of immunocompetent hosts with MCD (17, 73). Moreover, the intensity of KSHV viral load in peripheral blood seems to correlate directly with worsening symptoms and poorer prognosis in MCD patients (109).

1.7 Kaposi's Sarcoma-Associated Virus

1.7.1 Structure and Classification

The establishment of various PEL-derived cell lines that harbor KSHV has been pivotal in characterizing the virus. Based on genetic comparison of its major capsid protein, KSHV has been classified as a γ-2-herpesvirus, the first herpesvirus of the genus *Rhadinovirus* known to infect humans (231). As demonstrated by EBV and herpesvirus saimiri (HVS), the γ-herpesviruses have a predilection for infecting lymphocytes. EBV is well known to immortalize B-cells in vitro and is linked to AIDS-related lymphomas, endemic Burkitt's lymphomas, post-transplant lymphoproliferative disorders and Hodgkin's disease (73). HVS, a simian rhadinovirus, causes T cell lymphomas and can immortalize T cells in vitro. As discussed, KSHV is associated PEL and MCD, both lymphoproliferative diseases.

The family *Herpesviridae* has been divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* , and *Gammaherpesvirinae* (226, 228). The original classification was based on biological criteria and although quite good at predicting the relatedness of different herpesvirus species, herpesvirus taxonomy now relies on sequence information that provides solid data on gene organization and arrangement. The subfamily *Alphaherpesvirinae* includes human herpesvirus simplex type 1 and 2 (HSV-1, HSV-2) and varicella-zoster virus (VZV). These viruses have a short reproductive cycle, establish latency in sensory ganglia , and spread rapidly in culture. Betaherpesviruses include human herpesvirus 6 and 7 (HHV-6,HHV-7) and human cytomegalovirus (HCMV); they are characterized by slower spread in culture, a longer replicative cycle, and establish latency in lymphoreticular cells. The human gammaherpesviruses include Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). Generally, gammaherpesviruses have a very narrow host range and establish latency in lymphoid cells. Many of them will replicate *in vitro* in lymphoblastoid cells (227).

Herpesviruses all share structural features such as a linear double-stranded DNA genome, an icosadeltahedral capsid surrounded by an amorphous tegument, and a glycoprotein-rich envelope. Human herpesviral genomes range in size from 125kbp (65) to 230kbp (53). However, individual herpesvirus isolates may vary in genome size due to the number of internal or terminal repeat sequences. These repeats can also give rise to various genomic isomers (see Figure 1.2A). The HSV and HCMV genomes contain two sets of inverted repeats giving rise to four possible isomers depending on the orientation of the unique long (UL) and unique short (US)

sequences. VZV contains an internal repeat in opposite orientation to the terminal repeat, allowing two genomic isomers. The EBV and KSHV genomes contain direct terminal repeats and EBV also has smaller internal repeats. Within the herpesvirus genomes, seven blocks of conserved genes have been described and labeled the "herpesvirus core" that defines the herpesviruses as a family (53) (Figure 1.2B). This conserved core is generally located in the center of most of the human herpesviruses (53, 66). The seven blocks are not collinear between viruses but within the blocks the genes are homologous and collinear, although there is some divergence in gene number and relatedness. Many of the characteristic functions of herpesviruses are determined by products of the core genes, with the striking exception of the latency-associated genes which are not even conserved among the subfamilies.

As mentioned above, sequence data show that KSHV is a gamma-2-herpesvirus and as such is a member of the rhadinovirus subgroup of herpesviruses (26, 49, 193, 231). Like the "prototypic" rhadinovirus of New World primates, herpesvirus saimiri (HVS), the KSHV genome contains a central segment of low-GC DNA (L-DNA) that is flanked by repetitive high-GC DNA (H-DNA). The terminal repeats (TR) are tandem repeats of an 801bp H-DNA sequence (86% GC). The L-DNA contains over 80 open reading frames (ORFs); again like HVS, many of these encode unspliced homologues of host cell genes suggesting acquisition of host cDNA . Herpesviruses in general have done well at acquiring host genes, but the rhadinoviruses are particularly good pirates. Cellular homologues encoded by the rhadinoviruses generally function in immune evasion, nucleotide metabolism and

cell growth. Unlike other subfamily members, KSHV also encodes viral versions of IL-6, three CC chemokines, and interferon response factors (Figure 1.3). Although each rhadinovirus has acquired a slightly different subset of host genes, there is strong positional similarity with cellular homologues particularly prevalent near the genomic termini (for review see (194)).

KSHV has been further subclassified based on some of its more variable sequences. Zong *et al* described three strains, A, B, and C. Strain A was found more often in classic-Mediterranean KS, whereas B and C were found in African KS. Although strains B and C were found in AIDS-related KS cases in the U.S., most isolates from the east coast were nearly identical, leading the authors to postulate a common source. More recently, other investigators have described KSHV genotypes I-IV and found that those corresponding to types A and B of the earlier classification scheme are predominant in the U.S. There is no convincing evidence that specific strains are preferentially associated with disease type or severity (117).

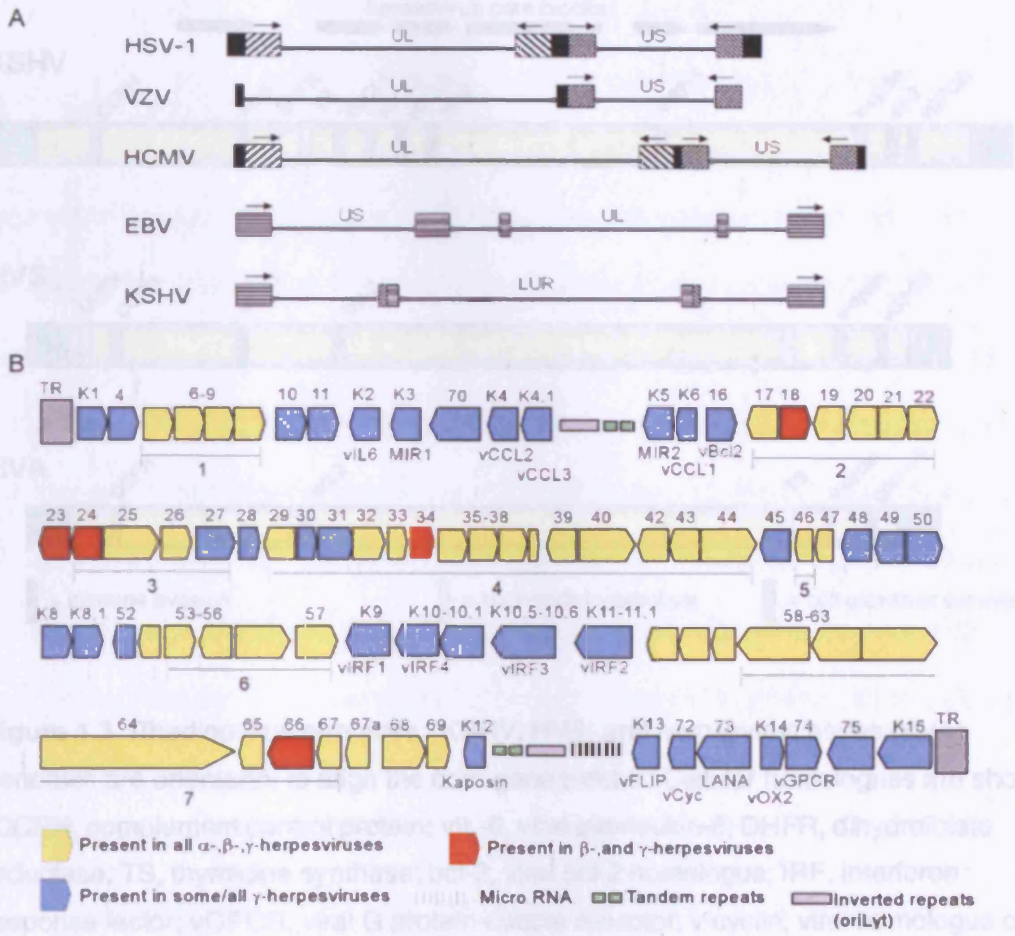

Figure 1.2 Genome structure of the human herpesviruses. **A**) HSV and HCMV both contain 2 sets of inverted repeats that result in 4 possible isomers with respect to the orientation of the unique long (UL) and unique short (US) sequences. VZV contains a large internal repeat that is repeated at only one terminus, giving rise to 2 possible isomers. The γ-herpesviruses EBV and KSHV have direct terminal repeats (TR) and in the case of EBV a set of internal repeats dividing the genome into UL and US sections. Both also have smaller internal repetitive sequences. Like all rhadinoviruses, KSHV has one long unique region (LUR) flanked by the direct TRs. Horizontal hatch represents direct repeats and diagonal hatch represents inverted repeats. **B**) Schematic of the KSHV genome; ORFs with homology to other herpesviruses are numbered while unique genes are prefaced with 'K'. Some of the unique KSHV genes discussed in this thesis are also labelled with more descriptive names. The seven conserved herpesvirus core blocks are numbered as shown. Adapted from references (193, 222, 231). Introns not shown for clarity.

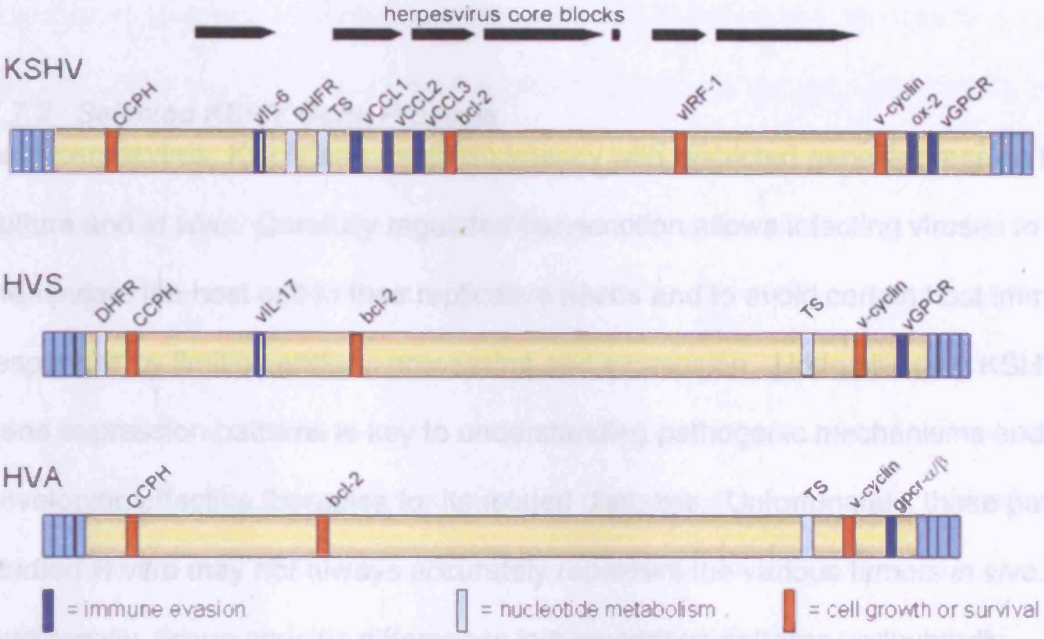

Figure 1.3 Rhadinovirus genomes. KSHV, HVS, and herpesvirus ateles (HVA) genomes are orientated to align the core gene blocks. Cellular homologues are shown. (CCPH, complement control protein; vIL-6, viral interleukin-6; DHFR, dihydrofolate reductase; TS, thymidine synthase; bcl-2, viral *bcl-2* homologue; IRF, interferon response factor; vGPCR, viral G protein-couple receptor; v-cyclin, viral homologue of D-type cyclins; vCCL-1,2,3, viral homologues of CC-type chemokines; gpcr-α/β, G protein-coupled receptor α/β.) Adapted from Neipel et al, 1997 (193). Genes are colour-coded for functional class.

1.7.2 Selected KSHV Gene Products

As a herpesvirus, KSHV can establish latency with restricted gene expression in cell culture and *in vivo*. Carefully regulated transcription allows infecting viruses to manipulate the host cell to their replicative needs and to avoid certain host immune responses by limiting antigen processing and expression. Understanding KSHV gene expression patterns is key to understanding pathogenic mechanisms and developing effective therapies for its related diseases. Unfortunately, these patterns studied *in vitro* may not always accurately represent the various tumors *in vivo*. Additionally, tissue-specific differences in transcription patterns undoubtedly contribute to the very disparate clinical presentations of KSHV-mediated disease. Despite these inherent difficulties, work in this area has allowed some generalizations.

Class I gene transcripts, or latent genes, are present at baseline in uninduced PEL-derived cell lines and KS spindle cells. These include the following: a D-type cyclin homologue; a putative apoptosis inhibitor vFLIP; transcripts from the K12 region (kaposins) of poorly understood function; and the latency-associated nuclear antigen, LANA (234, 257, 299). Class II transcripts are those present in low copy at baseline but increase with chemical induction of PEL-derived cell lines. Among these are homologues of interleukin-6, interferon regulatory factor, a G protein-coupled receptor, and two CC-type chemokines. Class III genes, generally well conserved among herpesviruses, encode structural proteins and although not expressed during latent infection, are inducible in PEL cell lines (234). In KS tissue,

only a small number of cells express Class II and III transcripts, representing lytic infection. The role of lytically infected cells in tumor maintenance and growth is debated. Furthermore, more detailed transcriptional analysis has shown complexities that while helping understand the pathogenic roles of various KSHV products, makes a simple class division of KSHV transcripts less tenable (263).

Many KSHV-encoded proteins certainly contribute to the pathogenesis of KS, PEL, and MCD. Some of the most suspicious are homologues of cellular genes involved in inflammation, cell cycle regulation, and angiogenesis. For example, KSHV encodes a homologue of the cellular D-type cyclins that is expressed in KS tumors and PEL cell lines (48). Like the cellular cyclin, the viral cyclin (vCYC) can activate kinases that phosphorylate and thereby inactivate pRB, a checkpoint protein that inhibits entry into S phase. Unlike its cellular counterpart, however, vCYC does not appear to be inhibited in the usual fashion, and can therefore sabotage a possible pRB-mediated defense against ongoing viral infection (264). By manipulating the host cell cycle to allow its own maintenance, KSHV may also contribute to tumorgenesis as has been seen in other lymphoid malignancies associated with dysregulated D-cyclin expression (184).

Apoptosis of infected host cells is an immunologic strategy that viruses must elude if they are to establish persistent infection. KSHV encodes a homologue of the cellular bcl-2 family members that consist of various pro- and anti-apoptotic proteins (57). This viral bcl-2 (vbcl-2) contains crucial conserved domains for interaction with other bcl-2 members and has been shown to inhibit apoptosis *in vitro* (57). Expression of vbcl-2 in KS increases with lesion stage, and in culture is

present at low levels during latent infection but can be chemically induced like other class II products (183, 236). Whether the role of vbcl-2 is solely to prevent apoptosis and allow viral synthesis is unclear. Its importance in the pathobiology of KS is supported, however, by clinical remission of KS after treatment with paclitaxel, a compound known to inhibit bcl-2 function (238, 280).

KSHV also encodes a FLICE-inhibitory protein (vFLIP) that may work to prevent TNFR-1 and Fas-mediated apoptosis. FLICE, or caspase-8, is a protease necessary for the apoptotic cascade used by the innate immune response to protect against viral infection. After apoptotic receptors such as TNFR-1 and Fas are activated, they bind via their death domains to intermediary proteins that subsequently bind and activate FLICE. FLIPs can also bind these intermediary proteins and hence compete with FLICE (286). Virally encoded FLIPs are found in other γ-herpesviruses but their transcriptional control appears to vary from KSHV (237). The importance of this anti-apoptotic strategy to KSHV-related disease is under study.

Interferons mediate part of the innate immune response to viral infection. KSHV encodes homologues of the human interferon regulatory factors (IRF), transcriptional factors that modify interferon-mediated effects (231). In vitro, the KSHV-encoded vIRF1 is functional and can inhibit interferon signaling. Furthermore, when stably transfected into fibroblast cell lines, vIRF1 can transform cells that in turn form tumors in nude mice (93). More recent work shows that vIRF can inhibit the anti-proliferative effects of interferon-α, again supporting a role in KSHV-mediated tumor formation (86). vIRF1 may also help regulate other viral

genes, such as vIL-6 (156). Ongoing studies of the transcriptional control and expression *in vivo* of vIRF will allow better estimations of its role in KS, PEL, and MCD. To complicate matters, however, it is now known that three additional KSHV open reading frames encode factors homologous to the cellular IRFs (36).

Another KSHV protein with pathogenic potential is a homologue of human IL-6, a cytokine with many hematopoetic and immune response functions. IL-6 is a growth factor for EBV-transformed B cell lines and its dysregulation is associated with polyclonal plasma cell abnormalities (118). Furthermore, IL-6 has been linked to the development of Castleman's disease (153). These properties make vIL-6 a suspicious player in the KSHV-related lymphoproliferative disorders. Indeed, KSHV vIL-6 expression is detectable in lymph nodes of KSHV-infected MCD patients and has been postulated to be partly responsible for the autoimmune complications found more often in such cases (206). Several groups have shown that vIL-6 is expressed to a much greater degree in PEL and MCD *in situ* than in KS lesions, implying a less prominent role for vIL-6 in KS relative to PEL and MCD (256)

Angiogenesis is important to any tumor growth and here again KSHV seems to have pirated some relevant cellular genes. Open-reading frame 74 of KSHV encodes a G protein-coupled receptor that is most homologous to the human interleukin-8 receptors, CXCR1 and CXCR2 (48). KSHV vGPCR is a more promiscuous receptor than its cellular homologue, as it signals constitutively *in vitro,* and can be further upregulated by several chemokines (9). When injected into mice, vGPCR-transfected rodent fibroblasts caused spindle cell tumors with prominent vasculature. The vGPCR was assessed specifically for angiogenic

properties, and it was shown that medium conditioned by vGPCR-transfected cells could induce angiogenesis in human endothelial cells. Angiogenesis was mediated by vascular endothelial growth factor, a protein well established as a growth factor for KS (13). The function of the vGPCR is the primary subject of this thesis and is discussed in more detail in sections 1.9-1.11.

Other KSHV-encoded products that may contribute to angiogenesis *in vivo* are two homologues of human macrophage inflammatory proteins, vCCL-I and vCCL-II (231). The vCCLs differ from human CCLs in that they are angiogenic in standard chick chorioallantoic assays (27). Interestingly, both vCCL-I and vCCL-II can inhibit HIV cell entry *in vitro*, as can certain human chemokines. More recently, vCCL-II has been shown to be an inhibitor of vGPCR (98). Why KSHV encodes an antagonist for its own GPCR is unclear but may prove to be an interesting aspect to the KSHV life cycle.

Aside from the above cellular homologues, KSHV encodes other genes that display potentially vital signaling and transforming properties. For example, the KSHV K1 gene occupies an analogous position to the HVS *STP* gene that is required for HVS-induced transformation. K1 can transform rat fibroblasts and when engineered to replace *STP* in HVS, the recombinant virus maintains the ability to cause lymphoma in marmosets (151). K1 has an ITAM motif that can activate cytoplasmic tyrosine kinases and mimic signaling by the B cell antigen receptor (151). KSHV K12, also known as kaposin, causes focal transformation when expressed in Rat-3 cells. Furthermore, these cells produce highly vascular, undifferentiated sarcomas when injected into athymic mice (185).

As we can see, the list of KSHV genes that affect cell growth, proliferation, inflammation, and angiogenesis *in vitro* is long. Studies using monoclonal antibodies to examine protein expression are emerging, but more are required to determine which KSHV products are important in each KSHV-related disease. Such studies may also explain the apparent functional redundancy within the KSHV genetic armamentarium.

1.8 Pathogenesis

Synthesizing what is known about KSHV and its pathogenic role in AIDS-related malignancies still requires a great deal of speculation. There are probably as many theories as investigators and many issues remain controversial. The complexity of these diseases is immediately apparent upon histologic examination of a Kaposi's sarcoma lesion. Unlike most tumors, KS lesions harbor a variety of cell types including a mixture of inflammatory cells, endothelial cells and the characteristic spindle cell. And as discussed, the KS spindle cell frequently does not display a transformed phenotype, typically loses KSHV infection in culture, and is dependent on various growth factors and cytokines. Yet somehow, in the right immunologic setting, tumor growth ensues and KSHV infection persists.

There is evidence that even prior to acquiring KSHV infection, all patients at risk for KS show $CD8^+$ T-cell activation and increased levels of Th_1 cytokines, including IFNγ (249). Ensoli *et al* have postulated that at least in the cases of African KS and AIDS-KS, those at risk may be under abnormally high chronic allogenic stimulation from recurrent infection (77). These same up-regulated

cytokines such as IL-1, IL-6, TNF, and IFNγ, are also produced within KS lesions and can cause endothelial cells to take on the KS spindle cell morphology (78). KS spindle cells in turn secrete factors that may act as autocrine or paracrine growth factors resulting in lesion expansion. For example, cytokine-induced spindle cells secrete bFGF and VEGF, which display autocrine and/or paracrine mitogenic effects, and act synergistically to induce angiogenesis, edema and KS-like lesions in animal models (76, 233).

There is little doubt that early KS is a cytokine driven event and that KSHV plays a central role. All early KS lesions contain KSHV, making KSHV appear necessary for KS lesion formation. Whether KSHV causes the inflammation present in KS lesions or, alternatively, is somehow recruited later to a pre-existing inflammatory lesion is still debated. Both circulating monocytes and B cells can harbor KSHV, and it has been recently shown that infection persists in culture in the presence of the same inflammatory cytokines that are up-regulated in at-risk patients (178). This raises the possibility that persistently KSHV-positive circulating cells, specifically monocytes, may carry KSHV to tissue lesions composed of activated endothelial cells and inflammatory cells that can support KSHV infection and spindle cell formation through the proper milieu of growth factors. Of note, lesional monocytes are more often lytically infected, perhaps producing viral gene products with potent paracrine effects. These infected monocytes may even be responsible for spreading progeny virus to other lesional cells (22). Such a sequence could explain the multifocality of KS. It may only be later in lesion

development that the KSHV gene products discussed above act to accelerate angiogenesis and growth, eventually resulting in a true clonal tumor (77).

If all KS hosts display similar immunoactivation, it remains to be explained why the various forms of KS have different clinical presentations. For example, AIDS-KS is a much more aggressive form than classic-Mediterranean KS. Part of the answer may lie in HIV-1-encoded Tat, a protein necessary to HIV-1 replication. In culture, extracellular Tat can cause IFNγ-primed endothelial cells to grow, proliferate and differentiate, forming tube-like structures. These cells also secrete known angiogenic and tumor growth factors. Tat acts synergistically with bFGF to cause these effects in endothelial and KS spindle cells. Furthermore, when bFGF and Tat are inoculated together, they cause KS-like lesions in nude mice (75). In all, the evidence is strong that HIV-1 Tat is an important co-factor in KSHV disease; and as Gallo points out, this may explain why KS is rare in African populations where KSHV infection is common but HIV-2, rather than HIV-1, predominates (92).

The contribution of immunosuppression to the pathogenesis of KS-mediated disease is still difficult to assess. Part of the problem is that the normal immune response to KS is not yet understood, although some initial cytotoxic T-cell response studies have been done (204). Certainly not all KS risk groups show obvious immunosuppression, but subtle acquired or genetic defects may remain undiscovered. PEL on the other hand, occurs more frequently in late stage AIDS, a profoundly immunosuppressed condition, although cases do occur in HIV-negative individuals. Moreover, the advent of highly active anti-retroviral therapy appears to

have decreased the incidence of KS and may be beneficially affecting the incidence and course of PEL.

Although KSHV infects B cells, what happens thereafter to result in the KSHV-related lymphoproliferative disorders is unclear. As discussed above, KSHV certainly shows potential to influence B-cell survival via vIL-6 and to counter IFN-mediated anti-proliferative effects via vIRF. However, the circumstances under which these proteins function *in vivo* are not known. For example, it is possible that more than one KSHV gene transcription program exists in latently infected lymphocytes. Furthermore, the mechanism of the switch from latent to lytic infection has not been worked out. Host immune surveillance may dramatically influence these transcription patterns and thus dictate the consequences of KSHV infection. Supporting a very important role for immunosuppression in KSHV-related disease, Min *et al* have shown that KSHV DNA increases in peripheral blood mononuclear cells (PBMC) of HIV-infected patients as $CD4^+$ cell count decreases (176). With rising viral load it is possible that KSHV-mediated lymphocyte proliferation increases. This may start to explain the aggressiveness of KSHV-positive MCD in HIV-positive patients. It would also be relevant to the development of PEL, a clonal malignancy that may arise from a background of vigorous B-cell proliferation (72).

A correlation between detectable KSHV DNA in PBMCs and immune surveillance may prove important in other ways as well. For example, it has not been ruled out that KSHV-mediated disease can result from viral reactivation. The possibility still exists that KSHV infection is much more common than suggested by the current detection techniques based on antigen expression and antibody

development. It may be that KSHV detection in the infected immunocompetent host requires more sensitive techniques, and that both KSHV disease and viral detectability become more likely with immunosuppression.

1.9 G Protein-coupled receptors

Before discussing the KSHV vGPCR in more detail, I will quickly review some general principles of GPCRs. Also known as 7-transmembrane domain receptors or serpentine receptors, GPCRs are the largest family of signal-transducing molecules. There are approximately 747 human GPCRs including 350 olfactory receptors, 30 additional chemosensory receptors, and 367 other physiological receptors (274). By some accounts, GPCRs are the target of up to 30% of all clinically available medications with annual sales of $US 47 billion in 2003 (31, 101). Some examples are shown in Table 1.1.

The amino terminus of GPCRs is situated extracellularly while the carboxy terminus sits intracellularly. The seven α-helices span the cell membrane and make up the transmembrane bundle which is arranged in a tight barrel-like structure with a tightly packed core (Figure 1.4). The transmembrane regions support the three extracellular loops and an N-terminal segment that act as the cell surface receptor. In addition to its topographical organization, post-translational modifications such as phosphorylation and glycosylation add to the structural complexity of GPCRs. Potential ligands include neurotransmitters, growth factors, hormones, calcium ions, and photons. Binding of ligand induces a relative shift in the positions of the transmembrane α-helices. Small ligands may bind within the transmembrane

bundle, whereas larger ligands bind the amino termini and extracellular loops. Ligand-induced movement of the α-helices causes conformational changes of the intracellular loops that activate the heterotrimeric GTP-binding (G) protein. An 'agonist' is a ligand that binds a receptor and shifts the receptor's equilibrium towards the activated state. An 'inverse agonist' is one that pushes a constitutively active receptor towards an inactive state, while an 'antagonist' reduces the action of any other ligand by inhibiting the orthosteric or primary binding site. Antagonists are further subdivided into surmountable and insurmountable; the former participates in competitive and reversible antagonism. The latter either participate in irreversible competition at the binding site, non-competitive antagonism, or indirect antagonism (195). Once the receptor-agonist complex occurs, changes in receptor conformation activate the associated G protein(s) which exchanges GDP for GTP and splits into two separate signaling effectors: the α-subunit and the βγ-subunit. There exist about 20 different α-subunits, 5 β-subunits, and 13 γ-subunits in the mammalian system (149). While inactive, GDP is bound to the α-subunit and the βγ-subunit stabilizes this conformation by binding tightly to the Gα-GDP enhancing the coupling of the inactive heterotrimer to the associated GPCR. Upon activation however, GDP release is greatly accelerated over the slow baseline rate and GTP replaces GDP. Both the α-subunit and the βγ complex have the capacity to regulate effector pathways such as phospholipase C (PLC), ion channels, or adenylyl cyclase (for review, see (285)). Normal G proteins contain an intrinsic GTPase activity that converts GTP back to GDP, resulting in reassembly of the α and βγ subunits (Fig 1.5).

Table 1.1 *Examples of Prescription Drugs that Target GPCRs*

Brand Name	**Generic Name**	**GPCR (s)**	**Indication**
Claritin	loratidine	histamine H_1	rhinitis/allergies
Zyprexa	olanzapine	serotonin 5-HT_2 and Dopamine	anti-psychotic
Risperdal	risperidone	serotonin 5-HT_2	anti-psychotic
Cardura	doxazosin	α-adrenoceptor	prostate hypertrophy
Tenormin	atenolol	β_1-adrenoceptor	hypertension
Zantac	ranitidine	histamine H_2	peptic ulcer
Cytotec	misoprostol	prostaglandin PGE1	ulcer
Imigran	sumatriptan	serotonin 5-$HT_{1b/1d}$	migraine
Serevant	salmeterol	β_2-adrenoceptor	asthma
Imodium	loperimide	opioid	diarrhea
Zoladex	goserelin	gonadotrophin-releasing factor	prostate cancer
Duragesic	fentanyl	opioid	pain
Cozaar	losartan	angiotensin II	hypertension
Atrovent	ipratropium	muscarinic	COPD

Adapted from McMurchie and Leifert, 2006

Figure 1.4. Cross-eyed stereoscopic view of sensory rhodopsin II from *Natronobacterium pharaonis*. Edman et al, 2002. PDB: 1 GUE. Image created with KiNG (http://kinemage.biochem.duke.edu). 3-D image of sensory rhodopsin II is shown as a representative GPCR. Cross your eyes, relax your vision until there are three images, then focus on the center image.

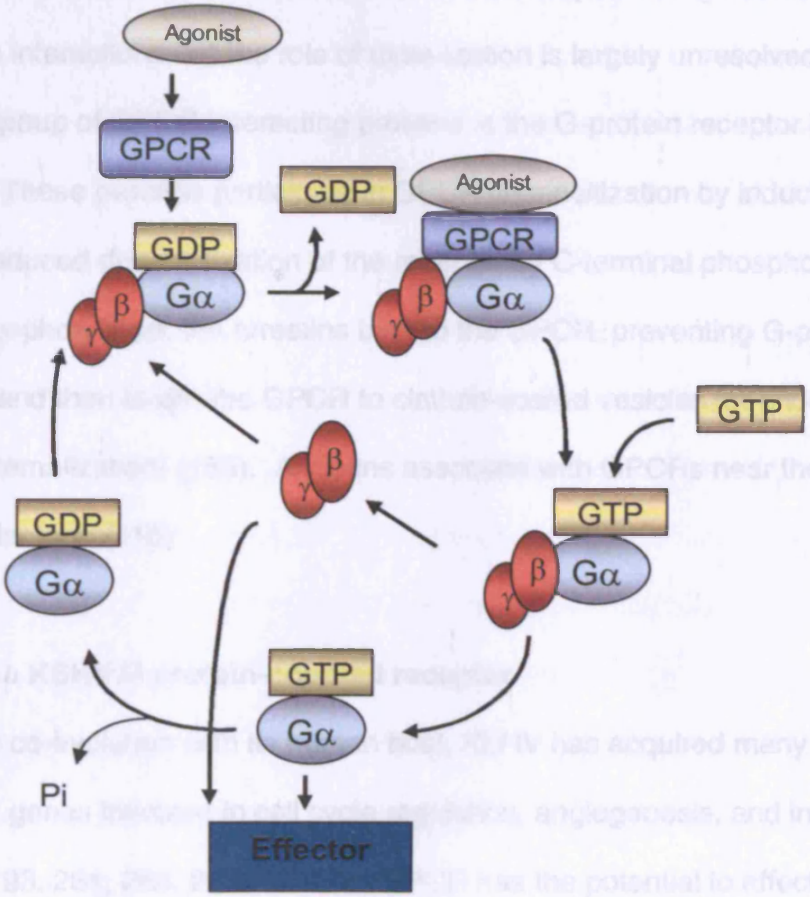

Figure 1.5 Schematic of GPCR activation. Upon ligand binding, the GPCR changes conformation and promotes release of GDP from the heterotrimeric G-protein and rapid exchange with GTP into the nucleotide binding site on the Gα subunit. The G-protein then dissociates into Gα and Gβγ subunits which interact with various downstream effectors. The signal is terminated by the spontaneous GTPase activity of the Gα subunit which hydrolizes GTP to GDP. (Pi, inorganic phosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GPCR, G-protein coupled receptor) *Adapted from R. Seifert, Nat Rev 2004 (242)*

In addition to G-proteins, GPCRs interact with other proteins that dictate receptor localization, trafficking, and ligand binding properties. For example, some GPCRs have been shown to form receptor dimers which may affect agonist recognition and G-protein interactions but the role of dimerization is largely unresolved (23). Another group of GPCR-interacting proteins is the G-protein receptor kinases (GRKs). These proteins participate in GPCR desensitization by inducing a rapid agonist-induced desensitization of the receptor by C-terminal phosphorylation (240). Once phosphorylated, the arrestins bind to the GPCR, preventing G-protein coupling and then target the GPCR to clathrin-coated vesicles for endocytosis (I.e. GPCR internalization) (155). Arrestins associate with GPCRs near the third intracellular loop (116).

1.10 The KSHV G protein-coupled receptor

During its co-evolution with its human host, KSHV has acquired many homologues of human genes involved in cell cycle regulation, angiogenesis, and inflammation (50, 57, 193, 231, 264, 286). KSHV vGPCR has the potential to affect all these processes. vGPCR has been characterized variably as an early or immediate early gene and is part of a bicistronic message that encodes K14/vOX-2 at its 5'-end (135, 187). (Cellular OX-2 or CD200 is expressed on the surface of many cell types and is postulated to provide an immune tolerizing signal. The role of vOX-2 is still unclear; for review, see (222)). The implications of vGPCR as a lytic gene will be discussed forther below. Like ORF 74 of murine gammaherpesvirus 68, KSHV vGPCR is a constitutively active variant of the human IL-8 receptors CXCR1, and

CXCR2, as well as herpesvirus saimiri ECRF3 (2, 9, 48, 111, 277). vGPCR was first shown to have oncogenic potential in fibroblasts, but later work also proved that vGPCR could immortalize human umbilical vein endothelial cells (HUVEC) and protect them from serum deprivation (13, 62, 179). In vivo, KSHV infects cells of endothelial and hematopoietic origin. Signaling molecules can have very different effects depending on the cellular context, so these initial studies in endothelial cells were important in establishing vGPCR as a vital component of KSHV-mediated cellular proliferation.

KSHV vGPCR can influence the expression of various cytokines involved in the biology of KS and primary effusion lymphoma. For example, KS depends on vascular endothelial growth factor (VEGF) for its highly vascular morphology and KS spindle cells secrete and respond in an autocrine manner to VEGF (169, 233). Sodhi, et al. show that vGPCR mediates VEGF secretion by stimulating transcription factor hypoxia-inducible factor (HIF)-1α in fibroblasts (252). Conditioned medium from vGPCR-expressing fibroblasts in tum stimulates endothelial cell growth and the switch to an angiogenic phenotype (13). A fascinating study by Bais et al. in HUVECs shows that vGPCR induces immortalization with constitutive VEGF receptor-2/ KDR expression and activation. This was associated with anti-senescence mediated by alternative lengthening of telomeres and an anti-apoptotic response (14). vGPCR expression also increases VEGF production from hematopoietic cells (37); this is significant in that VEGF is essential for PEL tumor growth and ascites production in mice and furthermore contributes to a more aggressive phenotype of MCD (6, 7, 197).

In addition to VEGF, vGPCR induces the expression of many pro-inflammatory cytokines important in KSHV-mediated disease. The monocytic line THP-1 expresses IL-1β, TNFα, and IL-6, and Jurkat cells elaborate IL-2 and IL-4 when transfected with vGPCR (241). In PEL cell lines, vGPCR upregulates KSHV vIL-6, an important growth factor in PEL and MCD (5, 127, 161). In endothelial cells, vGPCR upregulates the NFκB-dependent inflammatory cytokines RANTES, IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF). It also induces expression of the adhesion molecules VCAM-1, ICAM-1, and E-selectin. In the same study, supernatants from transfected KS cells activated NF-κB signaling in untransfected cells and caused the chemotaxis of monocytoid and T-lymphoid cells (208). This suggests that vGPCR may help recruit hematopoietic cells that make up the inflammatory component of KS lesions.

Although it is directly transforming in fibroblasts, and perhaps responsible for an immortalizing autocrine loop in endothelial cells (9, 13, 14), the ability of vGPCR to cause such robust and broad cytokine activity argues for an important paracrine role in KSHV-mediated disease. This is supported by the first vGPCR mouse model in which vGPCR was transcribed under the CD2 promoter (primarily T cell) and resulted in multicentric, angioproliferative lesions histologically similar to KS (291). The lesions ranged from erythematous maculae to vascular tumors; they contained spindle and inflammatory cells, expressed CD34, and VEGF. Despite causing endothelial cell tumors, vGPCR was expressed from relatively few infiltrating T cells.

KSHV vGPCR signaling may help explain why AIDS-associated KS is a more aggressive tumor than classic or iatrogenic KS. It has been known for several years

that the HIV-1 transactivator protein, Tat, activates KS cell growth and contributes directly to KS pathogenesis (16, 39, 74). Pati et al. have recently shown that HIV-1 Tat and vGPCR synergistically activate the transcription factor NFAT. In turn, NFAT activation is responsible for production of IL-2, IL-4, GM-CSF, and TNF-α as well as expression of ICAM-1, CD25, CD29, and Fas ligand by the T cell line HUT 78 (209). The expression of these surface molecules and inflammatory cytokines results in increased endothelial cell- T cell adherence and has important implications for vGPCR as a key molecule in the interaction between KSHV and HIV-1. Indeed, later work by the same group confirmed that HIV-1 Tat increases activation of NFκB and NFAT, and accelerates tumor formation in mice that are implanted with a cell line derived from a vGPCR transgenic mouse (112).

1.11 KSHV vGPCR Signaling and Function

1.11.1 Introduction

The signaling cascades vGPCR utilizes to effect changes in viral and host cell transcription are varied and in some cases cell-type specific. Despite increased understanding of its signaling potential, correlating these findings with biologic significance remains more problematic. In this section we will discuss what is known about the structure of vGPCR vis-à-vis its constitutive activity; the diversity of vGPCR signaling; as well as the potential roles in KSHV-mediated disease attributable to this unique receptor.

1.11.2 Multiple Ligands and Many Signaling Cascades

KSHV vGPCR is in the rhodopsin/β_2-andrenergic subfamily of GPCRs. The other two smaller subfamilies include the secretin-like receptors and the calcium-sensing/metabotropic glutamate receptors. Subfamilies are based on structural and genetic characteristics but share little intra-familial sequence homology. Unlike normal mammalian GPCRs, vGPCR signals on the absence of ligand. This constitutive activity is largely due to abnormal transmembrane helices two and three, as well as its cytoplasmic tail (121, 241). Additional mutational studies show that although the N terminus is required for ligand binding, it is not necessary for constitutive signaling (120, 229). Interestingly, despite its constitutive activity, vGPCR retains its ability to respond to various CXC and CC-type chemokines; these include agonists, inverse agonists, and antagonists. Agonists include IL-8 and growth-related oncogene-α (GROα) (9, 98-100, 111, 229). Antagonists include neutrophil-activating peptide-2 (NAP-2) and epithelial cell-derived neutrophil-activating 78 (ENA-78) (229). Inverse agonists include interferon-γ-inducible protein-10 (IP-10), stromal cell-derived factor-1 (SDF-1) and KSHV vCCL-II, a viral CC chemokine (98, 99). Of note, it is the angiogenic ELR-positive (Glu-Leu-Arg prior to the first cysteine) CXC chemokines that are vGPCR agonists, while the non-ELR chemokines are inverse agonists (260). This has implications for vGPCR function and is discussed below. Another mechanism by which vGPCR may be modulated *in vivo* is via the GPCR-specific kinases (GRK), particularly the family containing GRK-4, -5, and -6 (97, 215). GPCRs in an active state can be bound by GRKs that lead to interaction with arrestin and inhibition of G protein interaction.

The biologic importance of GRK-mediated downregulation of vGPCR remains to be studied.

KSHV vGPCR not only binds a huge array of ligands, but utilizes a surprising number of downstream effectors. These are cell-type specific so we will concentrate on the data in endothelial and hematopoietic cells since they are the natural targets for KSHV infection. vGPCR can signal via Gαi and Gαq in both cell types (38, 62). Studies in HeLa cells suggest that coupling to Gα13 and RhoA may also be possible but this remains to be confirmed in a biologically relevant cell type (244). The βγ subunits of both pertussis-sensitive (Gαi) and pertussis-insensitive α-subunits also mediate vGPCR signaling (38, 62, 179, 250). The result is the activation of several kinases involved in endothelial and B cell proliferation. For example, vGPCR-mediated activation of a Gαi-PI3K/Akt axis promotes endothelial cell survival; this is likely via activation of NFκB (62, 179, 208). Although NFκB is also activated in B cells, it is neither Gαi- nor PI3K/Akt-dependent but rather mediated by a non-pertussis sensitive G protein such as Gαq (38). vGPCR also activates the mitogen- and stress-activated protein kinases (MAPK, SAPK) involved in cellular proliferation, angiogenesis, and inflammation via both Gαq and Gαi G proteins (13, 37, 250). This results in additional transcription factor activation including AP-1, CREB, and hypoxia-inducible factor 1a (HIF1α); the latter has been shown to result in VEGF expression from COS-7 cells may well apply to endothelial and hematopoietic cells (252).

Nuclear factor of activated T cells (NFAT) is a transcription factor that acts in conjunction with AP-1 to enhance the expression of many proteins in the productive

immune response; furthermore, it is required for the calcium-mediated latent-to-lytic switch in KSHV transcription pattern (164, 301). vGPCR induces NFAT activity via combined interaction with Gαq and Gαi and that this is augmented by HIV-1 Tat (37, 209). Chiou et al. discovered that vGPCR upregulates transcription via the T1.1/PAN, K1, and LLP latency promoters of KSHV, while others show a vGPCR-induced increase in the lytic products ORF 50 and ORF 57 in latently infected PEL cells (37, 58). So in addition to its effects on host cell transcription, it is clear that vGPCR has important effects on KSHV transcription patterns; as discussed later, this has important implications for the role of vGPCR in the KSHV viral life-cycle.

1.11.3 KSHV vGPCR and Disease Pathogenesis

KSHV vGPCR is a promiscuous receptor that activates cell survival signaling cascades, results in the elaboration of various cytokines, and can affect KSHV transcription patterns. Any or all of these functions may be vital to the pathogenesis of KSHV-mediated endothelial and B cell hyperproliferative syndromes. Some of the first vGPCR studies showed it transforms fibroblasts (9). However, arguing for a directly transforming role in KSHV-mediated disease is difficult: unlike the latent transforming viral genes of EBV, vGPCR is expressed in early lytic phase (135, 187). A lytically activated host cell is destined to die in the process of new virion production, so to express a viral oncogene under such circumstances appears at odds with promoting an autocrine survival advantage. Some have argued that early vGPCR expression in endothelial cells may set up a self-perpetuating growth-promoting paracrine loop via upregulation of VEGF and VEGF receptors (14); this is a very enticing scenario but requires further investigation.

The bulk of evidence indicates that vGPCR has its most potent effects via a paracrine mechanism. KS and PEL tumors show that a relatively small subset of cells are KSHV-infected and even fewer express lytic genes such as vGPCR (72, 160, 257, 268). Likewise, vGPCR-driven KS-like tumors in mice result from vGPCR expression by a subset of tumor cells (113, 291). All KSHV-driven tumors display some level of lytic replication. Given the instability of KSHV infection in most cell types, this is probably required to maintain ongoing infection and new virion production (147). When expressed from these lytically activated host cells, vGPCR could provide growth-promoting cytokine expression; and as a chemokine receptor involved in cell migration, it could also recruit new infectable cells to the tumor microenvironment. Figure 1.6 summarizes some of the cytokines and cell adhesion molecules upregulated by vGPCR that could explain its paracrine-driven proliferative effect on both hematopoietic and endothelial cells.

Despite its obvious potential to affect the tumor microenvironment, if vGPCR is expressed only briefly during the early lytic phase, the question remains as to how relevant its effects are in vivo. However, some evidence suggests vGPCR can be expressed in a dysregulated way, outside the normal KSHV lytic program (251). For example, HIV Tat can increase the expression of vGPCR; and the Ganem lab showed that vGPCR transcription is upregulated by RBP-Jκ, a transcription factor and target of the Notch pathway (158, 292). If a sustained upregulation of vGPCR were to occur in an abnormal or abortive lytic phase, it becomes easier to reconcile its in vitro potential with tumorgenesis and effects on the viral life cycle. Interestingly, abortive lytic cycle progression in which a subset of lytic genes is

expressed has been shown in other herpesviruses. As discussed in Chapter 7, dysregulated vGPCR expression would have implications for our recent work. It opens up the possibility that dysregulated vGPCR expression leads to inhibition of full productive lytic replication. In this state of limbo between normal latent and lytic phases, vGPCR-mediated elaboration of angiogenic and mitogenic chemokines would have a more prolonged and biologically important effect.

Reconciling all the data on KSHV vGPCR into one well circumscribed function is not yet possible. In fact, current concepts in virology and tumorgenesis may require us to interpret vGPCR as having multiple seemingly discreet functions. It is clear, however, that KSHV has devoted it evolutionary energies toward tight regulation of vGPCR: its expression is restricted in that it is transcribed within the 3'-end of a bicistronic message; furthermore, KSHV encodes its own inverse agonist of vGPCR, vCCL2. Such fine tuning of vGPCR signaling suggests that vGPCR may play different roles at different points in the KSHV life cycle. Work by Dezube et al. shows that during early de novo KSHV infection of endothelial cells, vGPCR transcription fluctuates in a cyclic pattern every 48-72 hours consistent with viral replication (70). So in addition to maintaining a suitable tumor microenvironment as discussed above, it may be that vGPCR has yet another role in early infection: perhaps to establish successful latency or encourage the initial rounds of KSHV replication.

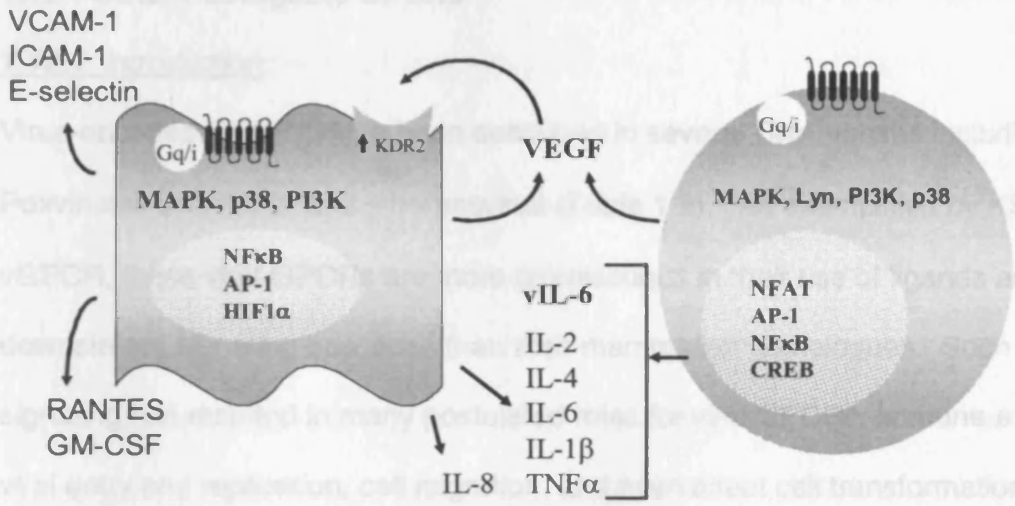

Figure 1.6. Summary of signalling pathway, cytokines and adhesion molecules upregulated by KSHV vGPCR. KSHV vGPCR is known to activate the MAP kinases, Src-family members and PI3-kinase depending on the cell type studied. Various growth and angiogenesis related transcription factors are also activated as are adhesion molecules that might play a role in manipulating interactions between endothelial cells, haematopoietic cells and other cell types in KS lesions. Cytokine elaboration is also influenced by vGPCR and may be involved in cell recruitment and/or immune evasion strategies. Various cell types have been used to study vGPCR; shown above are endothelial (left) and haematopoietic cells (right).

1.12 Other Pathogenic GPCRs

1.12.1 Introduction

Virus-encoded GPCRs have been described in several DNA viruses including Poxviruses and the β- and γ-herpesvirus (Table 1.2). As exemplified by KSHV vGPCR, these viral GPCRs are more promiscuous in their use of ligands and downstream signaling cascades than their mammalian homologues. Such broad signaling has resulted in many postulated roles for viral GPCRs: immune evasion, viral entry and replication, cell migration, and even direct cell transformation. In addition to virally-encoded constitutive GPCRs, several mutated mammalian GPCRs are also responsible for human disease.

1.12.2 Human Herpesviral GPCRs

EBV is a ubiquitous lymphotropic γ1-herpesvirus that infects and remains latent in over 90% of people by the time they reach middle age. Manifestations of acute infection often go unnoticed in children but can cause infectious mononucleosis in adolescents and adults. EBV infects the oropharyngeal epithelium and surface B cells of the tonsils; via the latter it disseminates throughout the reticuloendothelial system. Although infection persists throughout life, the immune-competent host generally suffers no long-term ill effects after the EBV-driven polyclonal B cell proliferation subsides; thanks in part to a vigorous cytotoxic T cell response. EBV can, however, contribute to more sinister sequelae: nasopharyngeal carcinoma is most prevalent in southeastern China; Burkitt's lymphoma is one of the most

common childhood malignancies in sub-Saharan Africa; post-transplant lymphoproliferative disorder is EBV-driven; and two-thirds of AIDS-related non-Hodgkin's lymphomas are EBV-positive.

Very recently the EBV open reading frame BILF1 was demonstrated to encode a functional, constitutively active GPCR that is heavily glycosylated and localizes to the plasma membrane in epithelial cell lines. The BILF1 signals via $G\alpha i$, but not $G\alpha q/11$ as evidenced by its ability to inhibit forskolin-stimulated CREB activity in a pertussis-sensitive manner in COS-7 cells. Like KSHV vGPCR, BILF1 appears to be an immediate-early product and can activate $NF\kappa B$. Interestingly, BILF1 reduces levels of phosphorylated RNA-dependent anti-viral protein kinase (PKR). PKR is an interferon-inducible enzyme with a role in intracellular anti-viral defense; BILF1 is the first GPCR shown to potentially inhibit this pathway (18, 210, 283). Further study of BILF1's role in the EBV life-cycle and its influence over host anti-viral responses are exciting prospects.

The β-herpesvirus family includes cytomegalovirus (CMV), human herpesvirus-6 and -7 (HHV-6, -7). In healthy hosts, acute CMV infection is a self-limited flu-like syndrome and by adulthood, 70-90% of people are latently infected. Infection during pregnancy, however, can lead to serious birth defects including blindness, deafness, seizures, and microencephaly. In the setting of AIDS, CMV causes retinitis, gastrointestinal disease, and various neurologic syndromes. In solid organ transplants, CMV causes hematologic disorders, hepatitis, pneumonitis and organ rejection. In bone marrow transplants the more common complications are graft-versus host disease, delayed engraftment, and pneumonitis.

The CMV genome encodes four GPCRs, two of which will be discussed here: US28 and UL33. Like KSHV vGPCR, HCMV US28 signals independently of an agonist but can be modified by CC chemokines and fractalkine (CX3CL1). Unlike KSHV vGPCR, US28 is located predominantly endosomally, not on the cell surface (278). Expression of US28 results in migration of smooth muscle endothelial cells and it is therefore postulated to play a role in viral dissemination and CMV-driven vascular disease (41, 137, 258). A role in immune evasion is also argued by virtue of the high turn over of US28 and the resultant sequestration of host-produced chemokines (24). As discussed for KSHV vGPCR, another important role for US28 may lie in its latent expression in hematopoietic cells, resulting in homing to sites of inflammation rather than lymph nodes (259). HCMV UL33 also demonstrates constitutive basal activity but binds no known chemokines. It is incorporated into viral particles and expressed on virus infected cells (166). UL33 activates CREB-mediated signaling and since many HCMV gene promoters contain CREs, it has been postulated that UL33 is involved in establishing viral infection or possibly reactivation (42). The rat and mouse CMV homologues of UL33 are important for replication in salivary glands and for general virulence, but similar studies are not available for HCMV UL33 (19).

Infection with HHV-6 and-7 generally occurs early in life. The former is the usual agent of exanthem subitum (also known as roseola infantum or sixth disease), a self-limited syndrome in children of fever and rash. Primary infection later in life causes an infectious mononucleosis syndrome much like EBV or CMV. HHV-7 is

Table 1.2 ***Human Herpesvirus G Protein-coupled Receptors***

Virus	Family	Gene	Constitutive	References
HCMV	β	U_S27	-	Fraile-Ramos et al., 2002
		U_S28	+	Kledal, et al., 1998
		U_L33	+	Marguiles et al., 1996
		U_L78	-	Bankier, DNA Seq., 1991 2;1
HHV-6	β	U12	-	Gompels et al., 1995 Isegawa et al., 1998
		U51	-	Milne et al., 2000
HHV-7	β	U12	-	Nicholas et al., 1996 Tadagaki et al., 2005
	β	U51	-	Nicholas et al., 1996 Tadagaki et al., 2005
EBV	γ1	BILF1	+	Beisser et al., 2005 Paulsen et al., 2005
KSHV	γ2	ORF 74	+	Bais et al., 1998 Rosenkilde et al., 1999

not convincingly associated with disease in the normal host, but like HHV-6 may become reactivated in the immuncompromised patient (60). Two ligand-dependent GPCRs with homology to the CC-chemokine receptors are encoded by both HHV-6 and HHV-7. U12 and U51 are positional and structural homologues of HCMV UL33 and UL78 (103, 125, 175, 196). HHV-6 UL12 binds RANTES, macrophage inflammatory protein 1α and 1β (CCL3, CCL4), and MCP-1 (CCL2); HHV-7 UL12 binds EBI1-ligand chemokine (ELC or CCL19), secondary monocyte chemoattractant lymphoid-tissue chemokine (SLC or CCL21), thymus and activation-regulated chemokine (TARC or CCL17), and macrophage-derived chemokine (MDC or CCL22) (188, 266). Both U12 homologues are functional and signal via non-Gαi pathways resulting in transient calcium flux. HHV-7 U12 induces migration of stably transfected Jurkat cells toward ELC and SLC, but not TARC or MDC. This leads the authors to postulate that HHV-7 U12 induces the migration of infected cells towards lymph nodes, perhaps facilitating viral transmission (266). Little is known of the function of HHV-6 U12.

In both HHV-6 and HHV-7, UL51 is expressed in the immediate-early and early stages post-infection. Both homologues bind many CC-type chemokines as well as the KSHV-encoded homologue of vCCL2. HHV-6 UL51 downregulates transcription of RANTES which may lend it an immunomodulatory role but this requires further confirmation. Even less is known about the function of HHV-7 UL51 (175).

1.12.3 Non-Viral Constitutively Active GPCRs and Disease

Aside from viral GPCRs, there exist several constitutively active GPCR mutants associated with human disease that may arise in germline or somatic cells (8, 61, 254). Familial forms of hyperthyroidism and hypoparathyroidism are due to mutations in the thyroid-stimulating hormone receptor in the former, and the extracellular domain of the calcium-sensing receptor in the latter (52, 273). The retinal degeneration of retinitis pigmentosa is thought due to mutations in the seventh transmembrane domain of rhodopsin, leading to over activation of photoreceptor cells (225). Mutation of the rhodopsin second transmembrane domain leads to congenital night blindness (220). A change in the sixth transmembrane helix of the luteinizing hormone receptor causes familial male precocious puberty as do other activating mutations (142, 243).

1.13 GPCR activation of protein tyrosine phosphatases

In Chapter 5 of this thesis I investigate the possible role of the non-receptor protein tyrosine phosphatases (PTPs) in conducting vGPCR-mediated signaling events. Important cell activities such as death, proliferation, and differentiation are regulated by signal transduction processes including protein phosphorylation and dephosphorylation; a balance of these is likely always in play. Although the protein tyrosine kinases (PTKs) are much better studied, the PTPs are likely as important and are increasingly the target of medicinal chemistry research (198, 216). Furthermore, very few studies on the interactions between GPCRs and PTPs have been performed. The PTPs will likely prove good candidates for rationally designed therapies and their role in GPCR signaling requires further investigation.

It has recently been estimated that 81 human genes encode PTPs, which is very similar to the number for PTKs(4). The non-receptor PTPs contain include SHP-1 and SHP-2 which contain tandem SH2 domains; an SH2 domain is a 100 amino acid motif that mediates binding to phosphorylated tyrosine residues on other proteins (141, 216). The SHP-1/2 SH2 domains are located near the N-terminus and are thought to bind intramolecularly to the PTP catalytic site thereby maintaining a basal inactive state. Once an appropriate phosphorylated tyrosine residue is encountered, however, an allosteric shift occurs activating the catalytic domain (for review, see (214)). SHP-2 is ubiquitously expressed, while SHP-1 is primarily found in haematopoietic cells (1, 293). SHP-2 has been implicated in many signaling pathways initiated by growth factors, including PDGF, EGF, IL-3, EPO, and GMCSF (122, 192). In some cases, SHP-2 appears to bind directly to certain receptors, but it also binds to many signaling intermediates such as p85, Grb2, Gab1, and Gab2. Despite its dephosphorylating activity, catalytically inactive dominant negative mutants of SHP-2 show that SHP-2 often functions in a positive way to enhance signaling; although it does negatively regulate JAK-STAT and p130 signaling (265, 295). It is also possible that in some cases, SHP-2 functions as a scaffolding protein and its phosphatase activity is not required to conduct a signal (163). Mouse studies have shown a role for SHP-2 in lymphoid, erythroid, and myeloid lineage differentiation (217, 218). SHP-1 on the other hand seems to negatively regulate haematopoietic cell growth and homozygous mutants develop systemic autoimmune disease associated with high levels of autoantibodies and excessive erythropoiesis (247, 248). Generally speaking SHP-1 has opposing

effects to SHP-2: SHP-1 attenuates signals from EPO, IL-3, and GM-CSF receptors and mediates inhibitory signals from γ Fc domains, TCR, BCR, and the NK inhibitory receptor (35, 55, 64, 294).

The most important phosphotyrosyl-containing motif that activates SHP-1 and SHP-2 is the immunoreceptor tyrosine-based inhibitory motif (ITIM). Once phosphorylated, variants of this consensus motif (I/V/L/S-x-Y-x-x-L/V) bind the N-terminal SH2 domain and activate the phosphatase catalytic site. The ITIM motif is also necessary to the inhibition, mentioned above, of B cell activation by the Fcγ receptor and the killer inhibitor receptor (KIR) of NK cells(63, 202).

We have noted that the KSHV vGPCR contains an ITIM consensus motif (LIYSCL) at the junction of the seventh transmembrane domain and the carboxy tail (see Figure 1.7). Interestingly, Duchene et al. have found that the bradykinin B2 receptor interacts directly with SHP-2 via an ITIM motif in the same location (71). Activation of the receptor resulted in increased SHP-2 phosphatase activity and which was necessary to the anti-mitogenic effects of bradykinin. There are, however, reports of GPCRs that activate SH2-containing PTPs in the absence of an ITIM. Furthermore, the angiotensin II receptor AT2 bears an ITIM but it is dispensable for SHP-1 activation (81, 157, 224, 255). Whether KSHV vGPCR activates the SH2-containing PTPs and if its ITIM motif is involved is addressed in this thesis.

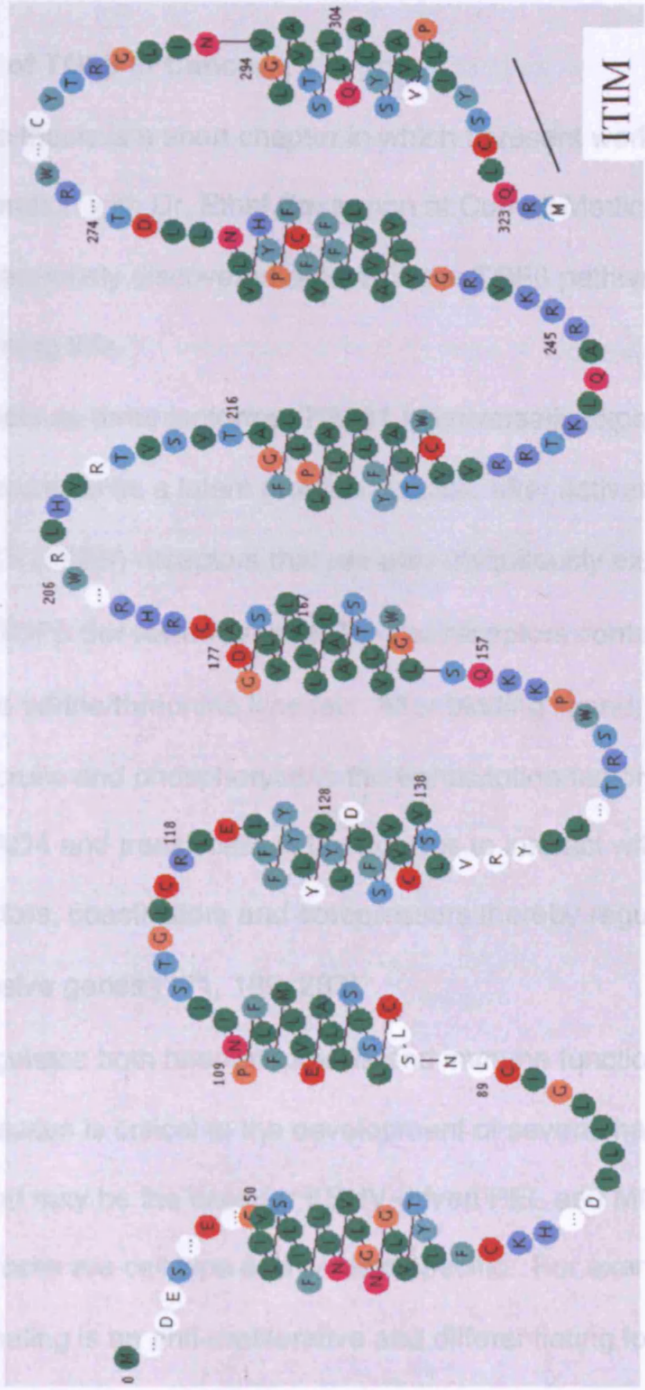

Figure 1.7. Schematic of KSHV vGPCR from GPCRDB data base. Shown is a schematic of KSHV vGPCR using single letter amino acid code. Note the seven transmembrane bundles. The amino terminus extends extracellularly while the carboxy terminus sits intracellularly. An ITIM consensus site LIYSCL is located at the junction of transmembrane domain 7 and carboxy tail. (http://www.gpcr.org/7tm/).

1.14 The role of TGFβ in Cancer

Chapter 6 of this thesis is a short chapter in which I present work I have done as part of a collaboration with Dr. Ethel Cesarman at Cornell Medical College in New York. We simultaneously discovered defects in the TGFβ pathway in PEL cells and are further exploring this.

TGFβ exists as three isoforms: TGFβ1 is universally expressed and the most abundant. It is secreted as a latent protein complex; after activation it binds to type I (TβRI) and type II (TβRII) receptors that are also ubiquitously expressed and have high affinity for TGFβ (for review, see (12)). The receptors contain cytoplasmic domains that are serine/threonine kinases. After binding ligand, TβRII recruits TβRI which in turn recruits and phosphorylates the transcription factors SMAD2/3. These in turn bind SMAD4 and translocate to the nucleus to interact with various transcription factors, coactivators and corepressors thereby regulating transcription of TGFβ-responsive genes (171, 189, 287).

TGFβ regulates both haematopoiesis and immune function; perturbation of both these processes is critical to the development of several haematologic malignancies and may be the case for KSHV-driven PEL and MCD. Effects on haematopoietic cells are cell type and context specific. For example, in progenitor cells, TGFβ signaling is an anti-proliferative and differentiating force. This maintenance of quiescence in early haematopoietic progenitors is less potent on later progenitors (87, 230). Via its effects on antigen presenting cells (APCs) and T cells, TGFβ has a predominantly immunosupressor role. It can block both the

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proliferation and maturation of T cells; autocrine TGFβ inhibits macrophage activation and promotes dendritic cell differentiation (25, 104, 132, 223).

Resistance to the homeostatic functions of TGFβ is common among haematologic malignancies. The most common mechanisms include downregulation of the TGFβ receptors or interference of signaling intermediates by oncoproteins such as Evi-1 and HTLV-1 Tax (146, 182). Solid tumours, on the other hand, more commonly acquire mutations of the receptors or SMADs (146). In this thesis we will see that PEL cells down-regulate TβRII and that exogenous expression of this molecule reconstitutes transcriptional and phenotypic responsiveness of PEL cells to TGFβ. Possible roles for KSHV-mediated downregulation of the TGFβ pathway will also be discussed.

CHAPTER 2: MATERIALS and METHODS

Standard techniques

2.1 Cell Culture

The PEL cell lines used were maintained in RPMI 1640 plus 40 mg/L gentamicin (Invitrogen) with 10% FBS (Atlanta Biologicals, Norcross, GA) at 37ºC, 5% CO2. HEK293, 293T, and NIH3T3 cells were maintained in DMEM plus 40 mg/L gentamicin (Invitrogen) with 10% FBS.

2.2 Agarose gel electrophoresis

Plasmids and DNA fragments were resolved on agarose gels to assess digestion and fragment sizes. Agarose concentration varied from 1-2% in 1x TAE. Ethidium bromide (Sigma) was added to agarose to stain DNA and a 1kB DNA size ladder (Invitrogen) was used to determine DNA sizes. Bands were visualized using a UV transilluminator.

2.3 DNA extraction

For gel purification, DNA bands were excised with a clean scalpel and purified using the QIAquick (Qiagen) extraction kit as per manufacturer's protocol. 30μl of water was used to elute DNA from the column. The same kit was used to clean up DNA produced by PCR reaction.

2.4 DNA ligation

DNA fragments were ligated in a volume of 20 μl at a molar ratio of 3:1, plasmid:insert. Ligation was performed at room temperature for 1 hour using DNA ligase (New England Biolabs).

2.5 Construction of tetracycline-responsive vGPCR-expressing plasmid

pTRUF2-Tet was constructed by initially cloning all the tetracycline regulatory elements into the cloning vector pSL301 (Invitrogen). The entire cassette was then transferred from pSL-301 to pTRUF2 (gift of Nicola Philpott, ICH, UCL) (302). In brief, pTet-tTS (Clontech) was digested with *Xho*I and *Hin*dIII and the resulting 2,098-bp tTS insert was blunted and ligated into the *Eco*RV site of pSL-301 to make pSL301-tTS. Next, pTet-On (Clontech) was digested with *Xho*I and *Pvu*II and the resulting 2,398-bp rtTA fragment was blunted and ligated into the *Sma*I site of pSL301-tTS to make pSL301-tTS-rtTA. Next, a simian virus 40 (SV40) pA tail was excised from pTRE (Clontech) by digestion with *Bam*HI and *Pvu*II; the fragment was blunted and ligated into the *Stu*I site of pSL301-tTS-rtTA to create pSL301-tTS-rtTA-pA. Next, the tetracycline-responsive promoter was excised from pTRE by digestion with *Xho*I and *Eco*RI, blunted, and ligated into the *Nru*I site of pSL301-tTS-rtTA-pA to create pSL301-tTS-rtTA-pA-TRE, which was then digested with *Pfl*MI and *Afl*II to remove the entire tetracycline-responsive cassette; the 5,886-bp fragment was blunted and ligated into the *Mfe*I site of pTRUF2 to create pTRUF2-Tet (12,168 bp). pTRUF2-Tet-vGPCR was made by digesting pcDNA3.1-*Sac*A, a plasmid derived from a genomic clone, with *Xba*I and *Eco*RI to excise the vGPCR ORF (48). The

vGPCR fragment was blunted and ligated into the blunted single *Nsi*I site in pTRUF2-Tet. Restriction enzymes were from Roche; T4 polymerase and the Klenow fragment used for blunting were from Fermentas.

2.6 Derivation of cell line BC3.6 and BC3.14

BC-3, an Epstein-Barr virus (EBV)-negative, KSHV-positive PEL line, was maintained in RPMI 1640 plus 40 mg of gentamicin (Invitrogen) per ml with 10% fetal bovine serum (Atlanta Biologicals, Norcross, Ga.) at 37°C under 5% CO_2 (10). BC-3.6 and BC-3.14 were established by electroporation of BC-3 with 10 µg of pTRUF2-Tet-vGPCR. The cells were allowed to recover for 48 h in full culture medium, and then 1 mg of G418 (Invitrogen) per ml was added. After 2 weeks under antibiotic selection, single cells were placed on irradiated fibroblast feeder layers (1 x 10^4 to 2 x 10^4 cells/well; 5,000 rads of cobalt $^{\cdot}$-irradiation) in 96-well plates by limiting dilution. After 2 weeks, wells with a single green fluorescent protein-positive colony were expanded and those with two or more were discarded. After reaching sufficient numbers, 48 lines were each screened for intact tetracycline inducibility-related protein expression by transfection, as described below, with 10 µg of pTRE-Luc (Clontech). Transfectants were split into two samples, one of which received 2 µg of doxycycline (Sigma) per ml. At 48 h, lysates were prepared and luciferase assays were performed. Lines with low background and high luciferase induction were used for vGPCR binding assays to assess the inducibility of vGPCR expression.

2.7 Transformation of competent bacteria

For most experiments competent *e.coli* used were TOP10 (Invitrogen). Bacteria were thawed on ice, DNA added and let mix with bacteria for 30 minutes, after which bacteria were heat shocked for 45 seconds at 42ºC. Fresh medium was added and bacteria allowed to recover for 60 minutes at 37ºC before plating onto agar plates supplemented with 100 μg/ml ampicillin.

2.8 Plasmid extraction and purification

For small scale extraction and purification of Plasmid DNA, alkaline lysis followed by affinity column capture was performed with the MiniPrep Kit (Promega) as per manufacturer's protocol. For larger scale production, alkaline lysis was followed by addition of isopropyl alcohol, centrifugation and resuspension in TE. Sample was then extracted with phenol:chloroform:isopropyl alcohol in a 1:1 ratio. After a 5 minute spin at 12000 rpm, the aqueous phase was removed and sodium acetate added to a final concentration of 0.3 M. 2.5 volumes of 100% ethanol were added to precipitate the DNA which was then pelleted, washed in 75% ethanol and then resuspended in TE.

2.9 Polymerase chain reaction

PCR was used to amplify DNA for cloning of ORFs, including the addition of epitope tags where required. It was also used in semi-quantitative RT-PCR experiments. PCR was performed in a 96-Plus thermocycler (MWG Biotech, UK). Expand High-

Fidelity DNA polymerase (Roche) was used when proof-reading was required. Otherwise, standard Taq polymerase (Roche) was used. Typical reaction mixture included 0.2 μm of sense and anti-sense primers (see Table 2.2), 5 μl of 10x buffer (containing $MgCl_2$), 200 μM dNTP, 0.5 units of polymerase, and DNA template in total volume of 50 μl. Parameters generally included 30-35 cycles at 95ºC for 1 minute, 55ºC, for 1 minute, and 72ºC for 3 minutes followed by final cycle of 72ºC for 10 minutes. For real-time PCR see 2.2.

2.10 Mutagenesis

To create vGPCR mutants Y314F and ALY, the QuikChange II Site-Directed mutagenesis Kit (Stratagene) was used. pKSHV-vGPCR was amplified using primers containing the desired mutations (see Table 2.2) for 12 cycles with an annealing temperature of 55ºC (1 minute) and an elongation temperature of 68ºC (6 minutes). After amplification, the product was digested for one hour at 37ºC to destroy the input pKSHV-vGPCR plasmid. One Shot Top Ten chemically competent *E. coli* were then transformed, colonies picked and grown the next day, followed by plasmid mini-preparation and sequencing by the Wolfson Institute Scientific Support Services Group.

2.11 Restriction digest analysis

Plasmids and/or DNA fragments were digested when necessary for cloning in a 10 μl reaction mixture consisting of 200 ng DNA, 1 ml of 10x buffer, and 0.5-1 units of

restriction enzyme. Reactions were incubated at 37ºC for an hour. All restriction enzymes were from Promega.

2.12 RNA extraction

Approximately 2-5 x 10^6 cells were homogenized in o.5 ml of TRI Reagent. RNA was extracted by adding 0.1 ml chloroform and mixing vigorously, followed by centrifugation at 12000 rpm for 15 minutes at 4ºC. To the aqueous phase was added 0.25 ml isopropanol, followed again by centrifugation as above. The pellet was washed in 75% ethanol, air dried for 10 minutes and then solubilised in 30 μl of water. Prior to use for reverse transcription, a 1 μg aliquot of RNA was treated for 1 hour at 37ºC with DNase 1 (Roche), followed by heat inactivation of the DNase 1 at 75ºC for 10 minutes in presence of 5 mM EDTA.

Cellular staining and analysis

2.13 Annexin V and PI staining

Cells were washed twice with ice-cold PBS and then suspended in 1x binding buffer (0.1 M HEPES pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of approximately 1 x 10^6. 100μl of suspension was transferred to a 5 ml culture tube to which 5 μl of annexin V-FITC (BD Pharmingen, cat# 556420) and 2 μl of PI (stock concentration 50 μg/ml) were added. After incubation for 15 minutes at room temperature in the dark, 400 μl of binding buffer was added and cells were analyzed

by flow cytometry. As controls for compensation adjustment, unstained cells and cells singly stained for PI or annexin V were also analyzed.

2.14 Immunofluorescence

HEK 293 cells were grown overnight on glass coverslips in 6-well plates. They were then transfected with plasmid constructs as shown in Figures. 48 hours post-transfection, cells were washed three times in cold PBS and blocked for one hour with 1% BSA in PBS. Cells were then incubated with anti-FLAG antibody (M2 clone, Sigma) at a dilution of 1:500 in PBS with 1% BSA for 1 hour in a humid chamber. Cells were then gently washed three more times in cold PBS followed by one hour incubation with a 1:60 dilution of anti-mouse-FITC antibody in PBS with 1% BSA. After three more washed in PBS, coverslips were inverted onto glass slides containing one drop of Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Images were obtained using an Axiovert 100 microscope, LD Acroplan 20x/0.04 numeric aperture objective, Axiocam camera, and Axiovision 3.0.6 software (all from Zeiss, Oberkochen, Germany).

2.15 Flow Cytometry

Forty-eight hours after transfection, HEK 293 cells were detached with a dilute Trypsin solution and washed in cold PBS. They were then resuspended in 50 μl of a 1:40 dilution of anti-FLAG antibody (M2 clone, Sigma) in PBS with 1% BSA and allowed to stand on ice for one hour. Cells were washed once in cold PBS and then stained with anti-mouse-FITC secondary antibody at a dilution of 1:60 in PBS, 1%

BSA for one hour. Cells were washed twice in cold PBS and resuspended in a volume of 100 μl of PBS. Ten thousand cells were then analyzed using a Facscalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ)

2.16 Cell Cycle Analysis

BC3.14 cells in exponential growth were incubated for 48 hours with or without doxycycline and then fixed with 70% ethanol overnight. Forty eight hours was chosen as the time point based on our previous work with BC3.14 showing that vGPCR signaling and phenotypic effects are not seen prior to 48 hours. They were then stained with propidium iodide at a final concentration of 50 μg/ml with RNaseA 100U/ml. When nocodazole (Sigma, St. Louis, MO) was used to inhibit mitosis, it was added for 17 hours to cells at a final concentration of 0.2 μg/ml. Ten thousand cells were then analyzed using CellQuest and ModFit on a Facscalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). To quantify S-phase, cells were incubated in the dark at 37ºC for 4 hours in the presence of 100 μM BrdU (Sigma). They were then plated using a Cytospin 2 (Shandon, Thermo Electron Corporation, Waltham, MA) and fixed for 30 minutes with 70% ethanol. Cells were washed in PBS and incubated for 15 minutes with 2N HCl to denature genomic DNA. After several more PBS washes, 70 μl of staining solution was added (50 μl PBS with 0.5% BSA and 0.5% Tween-20, 20 μl anti-BrdU-FITC (BD Pharmingen, Franklin Lakes, NJ)) and cells were incubated for 1 hour in a dark humid chamber. Cells were then washed and mounting medium with DAPI was applied (Vector Laboratories, Burlingame, CA). At least 100 cells were counted and S-phase

expressed as the percentage of FITC-staining cells relative to total number of DAPI-staining cells. Images were obtained using an Axiovert 100 microscope, LD Acroplan 20x/0.04 numeric aperture objective, Axiocam camera, and Axiovision 3.0.6 software (all from Zeiss, Oberkochen, Germany).

Transfections and Luciferase assays

2.17 Transfections

Transfections of BC3.14 were performed on exponentially growing cells by electroporation (Bio-Rad Gene Pulser II, Bio-Rad, Hercules, CA) at settings of 270 mV and 975 mF in 0.8 cm cuvettes (Invitrogen, Carlsbad, CA), using $8x10^6$ cells/cuvette resuspended in 0.8 ml RPMI 1640. Cuvettes were prechilled on ice, and after transfection, cells were quickly plated in full culture medium as described above. HEK293, 293T, and NIH3T3 cells were transfected in 6 cm dishes or 6-well plates (~40% confluent) using Fugene 6 per manufacturer's protocol (Roche Diagnostics).

2.18 Luciferase Assays

For BC3.14 luciferase assays, 10 μg of the reporter-luciferase construct were transfected and cells were then divided evenly and plated with or without doxycycline (Calbiochem, EMD Biosciences, Darmstadt, Germany). After 48 h, lysates were prepared using 1x Cell Culture Lysis Reagent as per the manufacturer's directions (Promega, Madison, WI). Assays were performed using

10 μl of lysate and 50 μl of beetle luciferin in a Fluoroskan Ascent FL (Thermo Electron), using a 10-s read time. Protein concentration was used for normalization and was determined by the Bradford method with Bio-Rad DC Protein Assay Reagent after diluting samples and standards 1:1 in PBS. Since cells were divided after transfection, transfection efficiency was inherently controlled for, and protein equalization was deemed sufficient.

Luciferase assays in HEK293 cells were performed 48 hours after transfection of 300 ng of Luciferase reporter construct (along with other constructs as indicated in figure legends). 15 ng of TK-renilla was included to control for transfection efficiency. Lysates were made at 48 hours using the Passive Lysis buffer included in the Dual Luciferase Kit (Promega). Assay was performed as described above with the addition of a 10-s read time for renilla values. All firefly values were normalized to renilla values to control for transfection and harvesting efficiency.

Protein Biochemistry

2.19 Immunoprecipitation and phosphatase assay

For immunoprecipitation experiments, HEK 293 cells were lysed in RIPA buffer 48 hours after transfection. 800-1000 mg of lysates was incubated overnight with 2 μg of the appropriate primary antibody followed by one hour incubation with Protein G-plus beads (Santa Cruz). The immunocomplex was washed three times in RIPA buffer and the pellet resuspended in 2x loading buffer. The complex was boiled for

2-3 minutes and loaded on 10% PAGE gels. Immunoblotting was performed as below.

In the case of immunoprecipated phosphatase assays, cells were lysed in CHAPS lysis buffer (50 mM Tris-HCL pH 7.5, 1.5% CHAPS, 150 mM NaCl, 5 mM EDTA, protease inhibitors). Prior to IP, 4 μg of anti-SHP2 antibody was incubated for 1 hour with 50 μl packed protein G-plus beads (Santa Cruz) in lysis buffer then washed to remove unbound antibody. 800 mg was then added to the antibody-bead complex and rotated at 4ºC for 2 hours. IP complexes were then washed twice in lysis buffer, twice in wash buffer (same as lysis buffer minus CHAPS), then twice in PTPase buffer (50 mM Tris-HCL pH 7.0, 1 mg/ml BSA. 5 mM DTT. IP complexes were then resuspended in 50 μl PTPase buffer with 0.2 mM phosphorylated peptide RRLIEDAEpYAARG (Raytide, Upstate) and incubated for 30 minutes at 30ºC in a shaker. IP complexes were then pelleted and 25 μl of supernatant was assayed for a molybdate :malachite green:phosphate reaction complex by adding 100 μl of malachite green solution (Upstate), incubating for 15 minutes, then reading absorbance at 620 nm. Values were compared to a standard curve to derive free phosphate concentration.

2.20 Western blotting

BC3.14 cells were plated in full growth medium at $4x10^5$/ml in a 24-well plate and subjected to 2μg/ml doxycycline for various time periods as indicated in figure legends. Cells were diluted to keep below 1.0 x 10^6/ml to minimize effects of crowding. Cells were lysed with standard RIPA buffer with 1 mg/ml each of

aprotinin, leupeptin, and pepstatin, 0.5mM PMSF, and 1mM each of $NaVO_4$ and NaF (Sigma). Protein was quantitated by the Bradford method and loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10–12% gels. Semidry transfer to PVDF (Millipore, Billerica, MA) was performed using transfer buffer, 48mm Tris, 39mm glycine, 0.037% SDS, and 20% methanol. Blots were probed with primary antibody (Ab) overnight at 41ºC. HRP-conjugated secondary Ab was added after washing and detected by an enhanced chemiluminescence system (ECL) (Amersham, Little Chalfont, UK).

Western blotting in the case of HEK293, 293T, and NIH3T3 cells was similar in that after transfection, cells were incubated for 24-48 hours and lysed in RIPA buffer as above. Protein separation, transfer and blotting procedures were the same as for BC3.14 cells.

2.21 Kinase Assays

BC3.14 cells were lysed in IP buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1mM DTT, 0.1% Tween-20, 10 mM β-glycerophosphate, 1 mM NaF, 0.1mM sodium orthovanadate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM PMSF); 800μg of protein was precleared and then incubated overnight at 4ºC with 2 μl of the appropriate anti-Cdk antibody and 20 μl of protein G plus-agarose beads (Upstate). Beads were then washed four times in IP buffer and twice in 50 mM HEPES with 1 mM DTT. Kinase reactions were carried out in 30 μl of kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 1 mM DTT, 10 mM β-

glycerophosphate) along with 10 μg substrate, GST-RbC-pocket (aa 773-928). Cold ATP to final concentration of 50 μM with 10 μCi of γ-^{32}P ATP (6000 Ci/mmol) was added and mixture incubated at 30ºC for 30 minutes. The reaction was stopped by addition of Laemmli buffer and brief boiling. The reaction product was loaded and run on a 10% SDS-PAGE gel, dried and exposed overnight. Blots were also incubated with anti-Cdk2 antibody to check for equal loading. Antibodies used included: anti-Cdk2(M2), anti-Cdk4(C22), anti-Cdk6(C21) (Santa Cruz).

For the in vitro Src kinase assay, cells were lysed with modified RIPA buffer (50 mM Tris (pH 7.5), 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton, 0.1% SDS, 0.5% DOC, 1 μM EDTA and protease inhibitors as above). 800 μg of lysate was incubated overnight at 4°C with 2 μg anti-Src antibody and 30 μl Protein G-Plus agarose beads (Santa Cruz). Beads were washed three times in lysis buffer and twice in kinase buffer (20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.1% NP-40, 0.1 mM $NaVO_4$). Kinase reaction was performed for 15 minutes at 30°C in 30 μl kinase buffer with 5 μM cold ATP, 3 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Amhersham). Reaction was stopped with loading buffer and boiled for 5 minutes, 35 μl of sample was run on 10% PAGE gel after which gel was fixed, dried and exposed to detect auto-phosphorylated Src. 4 μl of same product was used for Western using the same anti-Src antibody to control for total protein loaded.

2.22 Binding assay

BC-3 and its derivative cell lines, while growing in exponential phase, were subjected to various doses of doxycycline for 48 h. Aliquots (2 x 10^6 cells) were then washed in cold phosphate-buffered saline and resuspended in 200 µl of binding buffer (RPMI 1640 with 10 mg of bovine serum albumin (Sigma) per ml and 25 mM HEPES). Samples were incubated in duplicate for 2 h with 0.1 ng of ^{125}I-GROα (2,200 Ci/mMol) (Amersham) in a total of 400 µl of binding buffer, after which they were centrifuged through a 0.75-ml sucrose cushion (20% sucrose, 140 mM NaCl, 40 mM Tris [pH 7.6], 0.4% BSA) at 3,000 rpm (500 x *g*) for 5 min. Approximately 0.8 ml of supernatant was discarded, and the tubes were centrifuged again. The remainder of the supernatant was carefully removed, the tips of the tubes were cut off, and the contents were counted for radioactivity in a ˙ counter.

Real-Time quantitative polymerase chain reaction

2.23 Quantitative RT-PCR (qRT-PCR)

Total RNA from cells was treated with DNase I (RNase-Free, Ambion, Oxon, U.K.) before cDNA synthesis according to the manufacturer's instructions. cDNA was generated from 1 µg of total RNA by using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time qRT-PCR was performed on an ABI PRISM 7700 sequence detector (Applied Biosystems) by using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in duplicate, with triplicate nontemplate controls (NTCs) in a 25-µl PCR reaction. One microliter of cDNA was

used in a 25-µl PCR mixture containing 1x SYBR Green PCR Master Mix and 0.3 µM GAPDH primers (5'-GGA GTC AAC GGA TTT GGT CGT A and 3'-GGC AAC AAT ATC CAC TTT ACC AGA GT), or 0.5 µM of primers as shown in table 2.2. The Q-PCR technique is based on detecting the amplified PCR product by labeling with a fluorescent dye. The SYBR Green dye stains double-stranded DNA formed by the polymerase. To normalize SYBR Green signal between wells, an internal reference dye is incorporated into the PCR AMpliTaqGold buffer; its signal is independent of the PCR reaction. Normalized reporter signals (Rn) from reactions containing cDNA template (Rn+) and non-template (Rn-) controls are calculated before there is detectable SYBR Green fluorescence. The normalized reporter signals from the template and non-template controls are used to calculate ΔRn (Rn+ minus Rn-). ΔRn is then plotted on an amplification plot against cycle number to analyze amplification of target sequence. Increases of ΔRn above the initial baseline indicate generation of PCR product. To determine the threshold cycle (Ct), the cycle at which amplification of product becomes statistically significant, a threshold is calculated as the average standard deviation of ΔRn in the early reaction, multiplied by an adjustment factor. Ideally, the threshold should lie in the linear range of the plot. From the threshold, Ct is then determined.

Q-PCR is also simultaneously performed for the housekeeping gene GAPDH to normalize between samples. The Ct for GAPDH is calculated in the same manner as above. The difference between the target product Ct and the GAPDH Ct is called ΔCt. The ΔCt value for each sample is the then compared to one reference sample

to give ΔΔCt. The final value for the relative expression of a transcript is expressed as a negative power of2 ($2^{-\Delta\Delta Ct}$).

Unwanted primer dimerization can be detected when optimizing Q-PCR primers using a dissociation curve program. This denatures the cDNA and then heats the reaction from 60ºC to 95ºC over 20 minutes to induce annealing and thereby generates a fluorescent signal when 50% of the primers dissociate. The presence of more than one signal at different temperatures suggests formation of primer dimers.

2.24 Primer design for Q-PCR

Primers were designed using Primer Express® (PE Applied Biosystems, UK) based on default parameters and guidelines.

Lentivirus production and transduction

2.25 Lentivirus production

The lentiviral vectors used in this project are based on human immunodeficiency virus-1 (HIV-1) and were generated from the pHR'SIN parental construct (67). There is no HIV viral protein expression from this plasmid. The vectors are self-inactivating due to a deletion in the 3' HIV-1 LTR (303), so that only the internal promoter will be active in transduced cells. Broad tropism is obtained by generating vectors with the G glycoprotein of vesicular stomatitis virus (VSV-G) envelope (34). Confluent HEK 293T cells were split 1:5 into 10 cm dishes the day prior to transfection. Using Fugene 6, cells were transfected with 1 μg of plasmid p8.91

(expressing gag-pol), 1 μg pMDG (VSV-G expressing plasmid) and 1.5 μg of pHR-Sin-CSGW-ΔNot1 which expresses GFP. To insert an alternative expression cassette, the plasmid was digested with *Bam* H1 and Not 1 to release the GFP insert and the transgene of choice was ligated into the *Bam* H1-Not 1 site. The cell culture medium was changed the next day. Cell medium was collected 72 hours post-transfection and spun for 2 hours at 28000 rpm . The pellet was resuspended in serum-free medium. Virus was tittered by infecting 100,000 293T cells with 10 μl of concentrated virus. After 72 hours, cellular DNA was isolated and Q-PCR performed for both lentiviral DNA and GAPDH. A titer of copies of lentivirus per cell was then calculated.

2.26 Cell transduction with lentivirus

1-5 x 10^5 BC3.14 cells were resuspended in fresh medium to which was added 1 ml of lentivirus at an MOI of 5 genomes per cell. Both cells and virus were first incubated for 15 minutes with polybrene to neutralize surface charge and to enhance virus-cell binding. Cells were harvested for protein or cDNA 48-72 hours after infection.

Solutions for Standard Methods

1x TE	10mM Tris pH 7.5 1mM EDTA
TAE (50x)	242g Tris base 57.1ml acetic acid 100ml 0,5M EDTA
DNA loading buffer	60% (w/v) sucrose solution 0.1% (w/v) bromophenol blue
Protease Inhibitors	0.1mM NaF 0.1mM $Na_3 VO_4$ 2 mg/ml aprotonin 100 mg/ml PMSF
Laemmli Sample Buffer (4x)	250 mM Tris pH 6.8 0.02% (w/v) BPB 4% (w/v) 2-βME 8% (w/v) SDS 40% (v/v) glycerol

Solutions for Protein Biochemistry

RIPA lysis buffer	50mM Tris pH 8.0 150 mM NaCl 1% NP-40 0.1% SDS

Kinase assay buffer(10x)	500 mM HEPES pH 7.4
	100 mM Mg Cl_2
	10 mM DTT
ATP stock (100 mM)	60 mg ATP
	Adjust to ph 7.0 with NaOH
	Distilled water to 1 ml

Table 2.1 Cell lines used in this thesis

Cell line	ATCC #	Morphology	Organsim	Growth	Reference
HEK 293	CRL-1573	Epithelial	*Homo sapiens*	adherent	Graham FL et al, J Gen Virol 1977 36(1) p59
BC3	CRL-2277	Lymphoblast	*Homo sapiens*	suspension	Arvanitakis L etal, Blood 1996 (88)p2648
NIH-3T3	CRL-1658	Fibroblast	*Mus musculus*	adherent	Jainchill JL et al, J Virol 1969 4(5) p549

Table 2.2 Primer sequences used in this thesis

Primer	Use	Sense/ Anti-sense	Sequence
ITIM_Mut5'	mutation	S	CGTGGTTCCCCTGATATTCTCCTGCCTGGGATCCC
ITIM_Mut3'		A	GGGATCCCAGGCAGGAGAATATCAGGGGAACCACG
ALY_Mut5'		S	GCGTCAGTCTAGCGCTGTACCTCCTGGTGGCATATTCT ACGC
ALY_Mut3'		A	GCGTAGAATATGCCACCAGGAGGTACAGCGCTAGACT GACGC
NheFLAG vGCR5'	epitope insertion	S	GTGCTAGCCACCATGGACTACAAGGACGACGATGACAAGG CGGCCGAGGATTTCC
XhovGCR3'		A	TTCTCGAGCTACGTGGTGGCGCCGGACATGAAAGACTGCC TGAGG
RIIBam5'	cloning	S	ATGGATCCACCATGGGTCGGGGGCTGCTCAG
RIINot3'		A	TTGCGGCCGCCTATTTGGTAGTGTTTAGGGA
vGCR_seq	sequence	S	GGAAACATGACTGCAGACTG
vGCR_300		S	GGTATCTGCCTAAACTCGCT
vGCR300R		A	AGCGAGTTTAGGCAGATACC
T7 uni		A	TAATACGACTCACTATAGGG
p21_5'	qRT-PCR	S	ACACCTTCCAGCTCCTGTAACATACT
p21_3'		A	GAAACGGGAACCAGGACACAT
p27_5'		S	CGGTGGACCACGAAGAGTTAA
p27_3'		A	GGCTCGCCTCTTCCATGTC
p18_5'		S	GCACTTGGCTGCCAAAGAAG
p18_3'		A	TTCCGATGCCCCACATTG
p19_5'		S	AGCCCGCACTGGATTCCT
p19_3'		A	CATCAGGCACGTTGACATCAG
ORF 26_5'		S	GATTCCACCATTGTGCTCGAAT
ORF 26_3'		A	CCCAGTTGCTGAGGCACG
ORF 50_5'		S	CACAAAAATGGCGCAAGATGA
ORF 50_3'		A	TGGTAGAGTTGGGCCTTCAGTT
ORFK8_5'		S	CAAGCTCGCTGTTGTCAACC
ORFK8_3'		A	ATGACCGTTTCACAGACCGG
ORF 8.1_5'		S	ATCCCTGTGGCGCTCCTAA
ORF 8.1_3'		A	CGATACGTGGGACAATTGGC
GAPDH_5'		S	GGAGTCAACGGATTTGGTCGTA
GAPDH_3'		A	GGCAACAATATCCACTTTACCAGAGT
ORF 73_5'		S	TTGCCACCCACGCAGTCT
ORF 73_3'		A	GGACGCATAGGTGTTGAAGAGTCT
vGPCR_5'		S	GTGCCTTACACGTGGAACGTT
vGPCR_3'		A	GGTGACCAATCCATTTCCAAGA
RIIsybr5'		S	TTGACTGAGTGCTGGGACCAC
RIIsybr3'		A	CTCACTGAAGCGTTCTGCCAC

Table 2.3 Primary antibodies for immunoprecipitation and immunoblotting

Antibody (clone/cat #)	Isotype	Company
cdk2 (M2)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
cdk4 (C-22)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
cdk6 (C-21)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
Mdm2	Mouse IgG	Santa Cruz Biotechnology CA, USA
FLAG (M1)	Rabbit IgG	Sigma
β-actin (Ab-1)	Mouse IgG	Oncogene
p21 (F-5)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
p53 (FL393)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
p27 (C-19)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
cyclin A (H-432)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
cyclin D2 (C-17)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
SHP-2 (C-18)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
SHP-1 (C-19)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
Gab1 (CT)	Rabbit IgG	Upstate
AKT (9272)	Rabbit IgG	Cell Signaling Technology
pAKT (ser473)	Rabbit IgG	Cell Signaling Technology
pSHP2 (Tyr542)	Rabbit IgG	Cell Signaling Technology
pSHP2 (Tyr580)	Rabbit IgG	Cell Signaling Technology
pERK42/44	Rabbit IgG	Cell Signaling Technology
ERK42/44	Mouse IgG	Upstate
pFAK (Tyr397)	Rabbit IgG	Upstate
pTyr (PY99)	Mouse IgG	Santa Cruz Biotechnology CA, USA
FAK (4.47)	Mouse IgG	Upstate
BrdU-FITC	Mouse IgG	BD Pharmingen
p18 (M-168)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
P19 (C-20)	Rabbit IgG	Santa Cruz Biotechnology CA, USA
p130 (AB-2)	Mouse IgG	Neomarker, CA
TGFβ (AB-100-NA)	Rabbit IgG	R&D Systems
v-src (Ab-1)	Mouse IgG	Oncogene

Table 2.4 Secondary antibodies for immunoblotting

Antibody (clone/cat #)	Isotype	Company
Anti-mouse-HRP (sc-2060)	IgG	Santa Cruz Biotechnology CA, USA
Anti-rabbit-HRP (PO217)	IgG	DAKO

CHAPTER 3:

3.0 Introduction

As discussed at length in the *Introduction*, KSHV vGPCR signaling properties and effects on cytokine production have been studied in various cell types, including common laboratory cell lines and primary endothelial cells. Unfortunately, little is known about the signaling properties of vGPCR in the haematopoietic cells relevant to KSHV-mediated disease. Haematopoietic cells serve as a reservoir and source of new KSHV virions in vivo; they therefore play a vital role not only in the KSHV lymphoproliferative disorders, but in KS as well. Furthermore, given the importance of cellular context to most signaling proteins and pathways, understanding of the contribution of vGPCR to KSHV-mediated pathobiology requires study of such cells. With this in mind, and just prior to my arrival at UCL, I derived KSHV-positive PEL cell lines engineered to express vGPCR in a controllable manner. Transient constitutive expression of vGPCR proved detrimental to PEL cells, so an inducible system was required. A single plasmid was designed to incorporate the sequences necessary for tight tetracycline inducibility along with the inverted terminal repeat (ITR) sequences from adeno-associated virus type 2 (AAV-2) to maximize the probability of stable integration (see Fig. 3.1)(105, 106). During latent AAV-2 infection, the AAV Rep protein binds DNA in or near the ITRs and mediates site-specific integration of the AAV genome into chromosome 19 of the human genome. However, even in the absence of Rep, the hairpin structure of the ITRs is thought to enhance random stable integration (85, 165, 213). Taking advantage of this

property, I established inducible cell lines with one round of transfection followed by clonal selection for tight vGPCR inducibility. A PEL cell line was chosen as the parental line to express vGPCR in the context of natural KSHV infection and the presence of the entire viral genome. This allowed us to create a model for assessing vGPCR-mediated effects on other KSHV genes. Since vGPCR is an early lytic gene, latently infected PEL cell lines express very low levels (see Fig. 3.2). Therefore, using this inducible system, we can selectively induce vGPCR expression without treating the cells with agents that induce the entire lytic viral cycle.

The vGPCR-inducible PEL cell lines are ideal tools for studying vGPCR signaling in the context of KSHV infection. One of the first things I noticed when performing signaling studies on these cells was that the expression of vGPCR inhibited cell growth, without significant cell death as assessed by trypan blue staining (see Fig. 3.2B). This effect was unexpected given that vGPCR had been characterized as a viral oncogene in NIH3T3 cells and has growth- and angiogenesis-promoting effects in endothelial cells. I therefore sought to describe more completely this apparent vGPCR-mediated growth inhibition in these KSHV-infected haematopoietic cells.

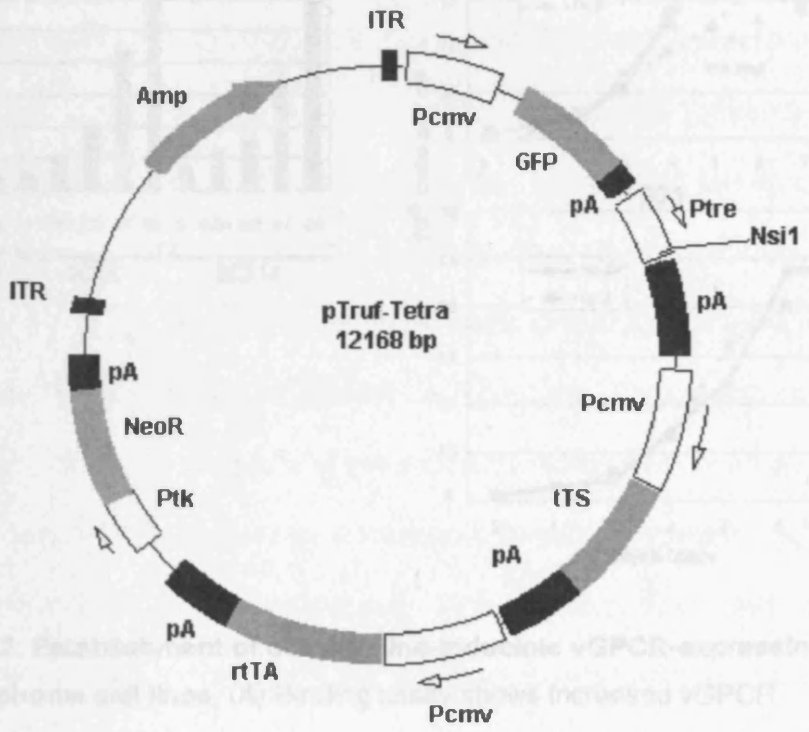


Figure 3.1. Map of pTruf-Tet, a single plasmid designed to allow tetracycline-mediated induction of transgenes cloned into the unique *Nsi*I site. ITR, inverted terminal repeat from AAV-2; Pcmv, CMV-derived promoter region; GFP, green fluorescent protein; pA, polyadenylation site from SV40; Ptre, tetracycline-responsive promoter; tTS, transcriptional silencer; rtTA, reverse tetracycline-responsive transcriptional activator; Neo^r, conveys neomycin resistance

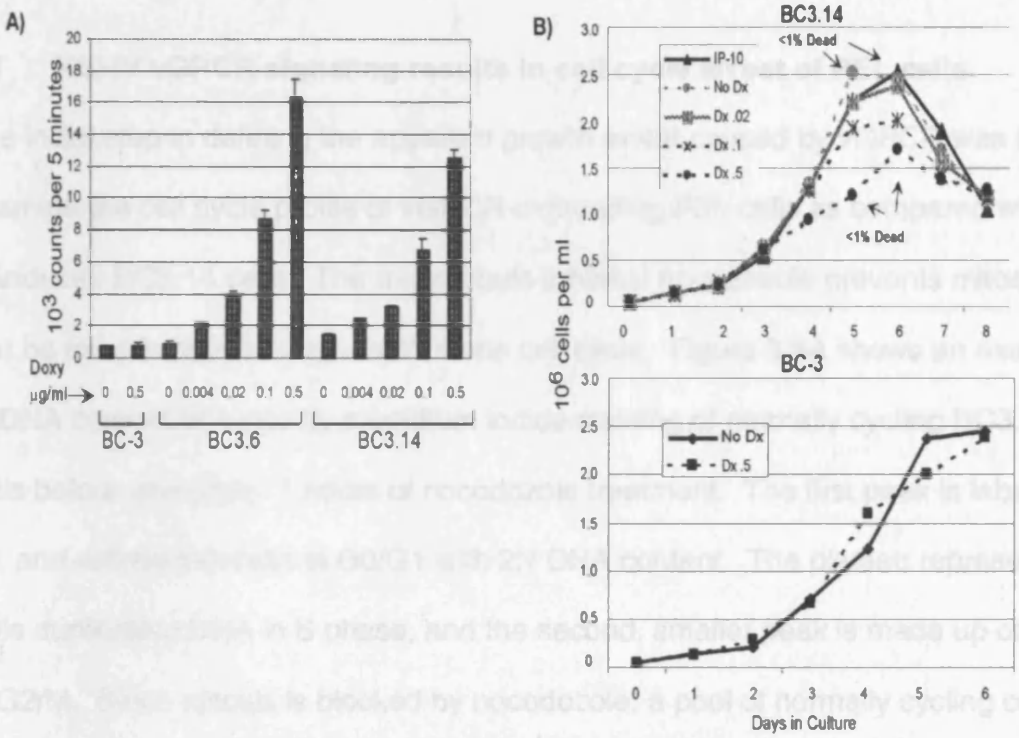


Figure 3.2. Establishment of doxycycline-inducible vGPCR-expressing PEL lymphoma cell lines. (**A**) Binding assay shows increased vGPCR protein expression with increasing doses of doxycycline. Cells were incubated with ^{125}I-GROα, and pellets were counted in a γcounter. The first two lanes verify that doxycycline has no independent effect on control BC-3 cells. (**B**) vGPCR expression slows the growth of PEL cells in culture. Cells were plated at 5 x 10^4 cells/ml with the doxycycline doses shown (in micrograms per milliliter) and counted daily by trypan blue exclusion. (Top) BC3.14 with increasing doxycycline doses and thereby increasing vGPCR expression. The percentage of dead cells does not vary between vGPCR-expressing and -nonexpressing cells. (Bottom) Control BC-3 cells incubated with 2 μg of doxycycline per ml show that doxycycline has no independent effect on cell growth. Shown is average of three independent experiments; error bars are omitted for clarity.

CHAPTER 3: RESULTS

3.1 KSHV vGPCR signaling results in cell cycle arrest of PEL cells

The initial step in defining the apparent growth arrest caused by vGPCR was to examine the cell cycle profile of vGPCR-expressing PEL cells as compared with uninduced BC3.14 cells. The microtubule inhibitor nocodozole prevents mitosis and can be used to take a 'snap-shot' of one cell cycle. Figure 3.3A shows an example of DNA content analysis by propidium iodide staining of normally cycling BC3.14 cells before and after 17 hours of nocodozole treatment. The first peak is labeled M1 and reflects the cells in G0/G1 with 2N DNA content. The plateau represents cells duplicating DNA in S phase, and the second, smaller peak is made up of cells in G2/M. Since mitosis is blocked by nocodozole, a pool of normally cycling cells will accumulate in G2/M over several hours. Figure 3.3B shows combined results of two such studies in BC3.14 cells with and without pre-incubation with doxycycline to express vGPCR. Cells were inoculated at identical concentrations and then incubated as normal for 48 hours with or without the doxycycline. Nocodazole was added for 17 hours and cell cycle position determined by propidium iodide staining and flow cytometric analysis (FCM). Results show that at baseline, BC3.14 cells progress rapidly through S phase and accumulate in G_2/M in the presence of nocodazole. However, when vGPCR is expressed, the cells arrest. Since the DNA profile does not change dramatically after the addition of nocodozole, this suggests that arrest is occurring throughout the cell cycle.

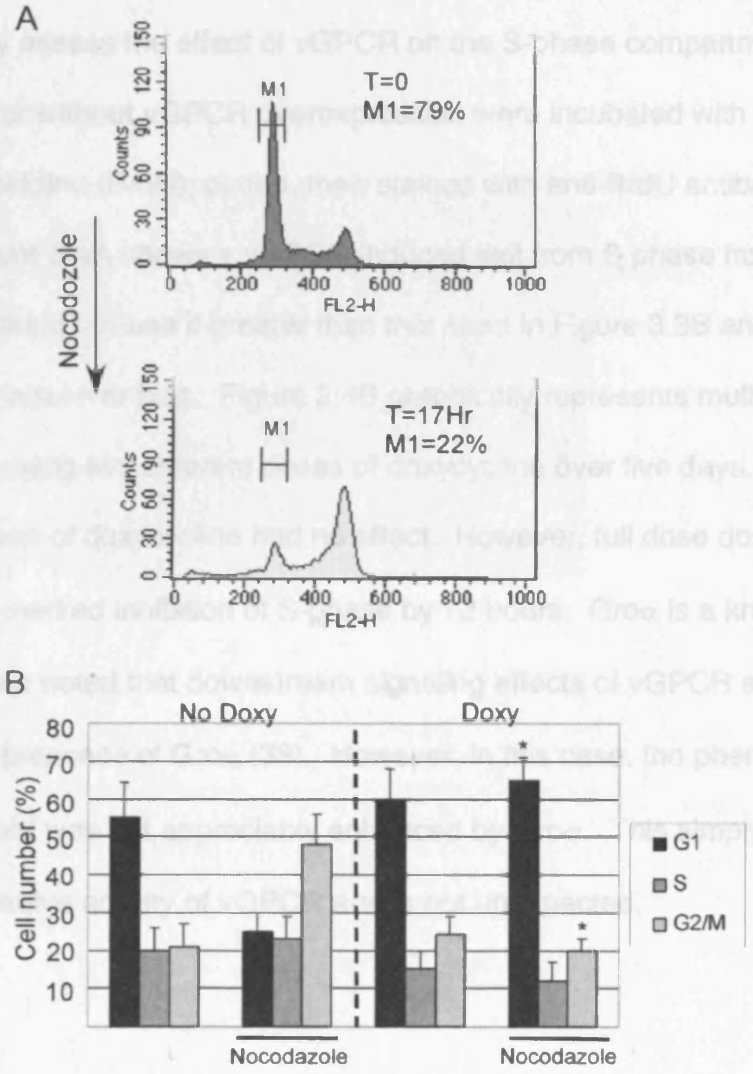


Figure 3.3. KSHV vGPCR expression results in cell cycle arrest. (**A**) BC3 cells were exposed to 0.2 μg/ml nocodozole for 17 hours to block mitosis. Cells were then stained with propidium iodide and analyzed by FCM. The percentage of cells in G0/G1 is shown as M1 fraction. (**B**) BC3.14 cells were exposed to 2 μg/ml doxycycline where indicated for 48 hours to induce vGPCR expression. Cells were then stained with propidium iodide and analyzed by FCM as in (A). The percentage of cells in each cell phase are shown. Average of two independent experiments. *, $p \leq 0.05$ compared to corresponding point in 'No Doxy' panel.

3.2 KSHV vGPCR inhibits entry into S-phase of PEL cells

To accurately assess the effect of vGPCR on the S-phase compartment over time, BC3.14 with or without vGPCR overexpression were incubated with bromodeoxyuridine (BrdU), plated, then stained with anti-BrdU antibody and counted. Figure 3.4A shows a vGPCR-induced exit from S phase from 50% to less than 20%. This decrease if greater than that seen in Figure 3.3B and may be explained by observer bias. Figure 3.4B graphically represents multiple such experiments using two different doses of doxycycline over five days. As suspected, a very low dose of doxycycline had no effect. However, full dose doxycycline resulted in a marked inhibition of S-phase by 72 hours. Groα is a known vGPCR agonist and we noted that downstream signaling effects of vGPCR are easer to detect in the presence of Groα (38). However, in this case, the phenotypic effect of cell cycle arrest was not appreciably enhanced by Groα. This simply attests to the strong constitutive activity of vGPCR and is not unexpected.

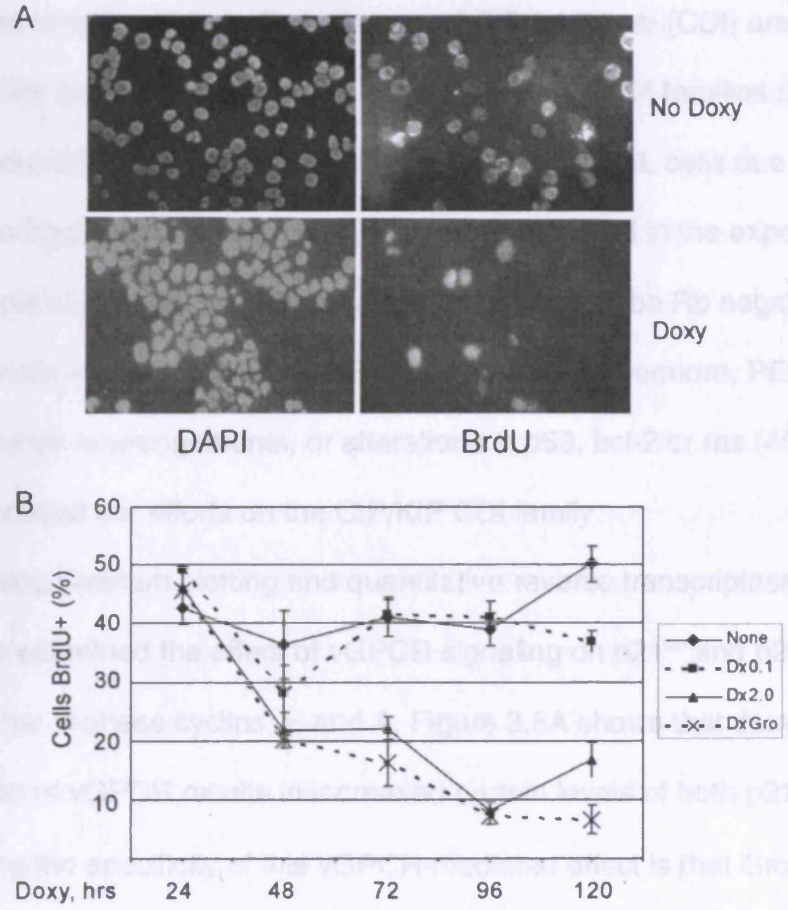


Figure 3.4. KSHV vGPCR inhibits S-phase entry in PEL cells. (A) BC3.14 cells were exposed to doxycycline where indicated for 48 hours and then fixed to glass slide by cytocentrifugation. Cells were incubated with BrdU for 6 hours followed by FITC-conjugated anti-BrdU. (**B**) BC3.14 cells were exposed to doses of doxycycline as shown for up to 120 hours. Cells were re-fed every 48 hours to prevent density-induced arrest. BrdU staining was performed at each time point as described above. Cells were manually counted and average of three experiments shown. Error bars

3.3 vGPCR causes a p53-independent transcriptional upregulation of $p21^{cip}$ in PEL cells

Cell cycle progression is regulated through a precise temporal activation of cyclin dependent kinases (Cdks). Two classes of Cdk inhibitors (CDI) are crucial to this ordered Cdk activation and include the CIP/KIP and INK4 families (246). Of note, endogenous p16INK4a expression is not detected in PEL cells due to either deletion or hypermethylation; BC-3, the parent line used in the experiments here is an example of p16 deletion. It has also been shown to be Rb negative, a characteristic of some but not all PEL lines (212). Furthermore, PEL cells do not display c-myc rearrangements, or alterations in p53, bcl-2 or ras (45). Therefore, we initially focused our efforts on the CIP/KIP CDI family.

Using Western blotting and quantitative reverse transcriptase-PCR (qRT-PCR), we examined the effect of vGPCR signaling on $p21^{cip}$ and $p27^{kip}$, CDIs known to inhibit the S-phase cyclins, E and A. Figure 3.5A shows that doxycycline-induced expression of vGPCR results in increased protein levels of both p21 and p27. Supporting the specificity of this vGPCR-mediated effect is that Groα results in slight enhancement of protein accumulation when added to vGPCR-expressing cells but has no independent effect. It is possible that accumulation of p21 and p27 merely reflect an enhanced accumulation of cells in G0/G1. We therefore used qRT-PCR to look for transcriptional upregulation of the CDIs. Figure 3.5B shows a clear increase in p21 message that would suggest a causal effect on cell cycle arrest rather than a secondary accumulation. Changes in $p27^{kip}$ transcript are not significant in the absence of Groα stimulation. Since cell cycle arrest does not

require the addition of a vGPCR agonist (see Figure 3.3), we conclude that vGPCR signaling mediates an increase in p21 but that increases in p27 are a secondary effect of cell cycle arrest and not likely to be an important part of the vGPCR signaling pathway. Furthermore, using qRT-PCR we assayed for p18 and p19, but found no detectable effect of vGPCR on the INK family of CDIs (Fig. 3.6A). As mentioned above, BC3 cells, the parent line of BC3.14, have been shown to express no p16 (212) and our experiments confirmed this for BC3.14 (data not shown).

Increases in p21 can be either dependent or independent of the tumor suppressor p53 (3, 96, 136, 297). We therefore assessed the vGPCR-mediated effect on p53 in our PEL cell line. Figure 3.6B shows that despite prolonged incubation with doxycycline, the resultant vGPCR expression did not result in accumulation of p53 or mdm2 as would be expected with p53 activity (15, 289). Furthermore, luciferase reporter assays using a p53-responsive promoter show some decrease in p53 activity (Fig. 3.6C). The cause of this decrease is unclear but certainly argues against a p53-mediated increase in p21.

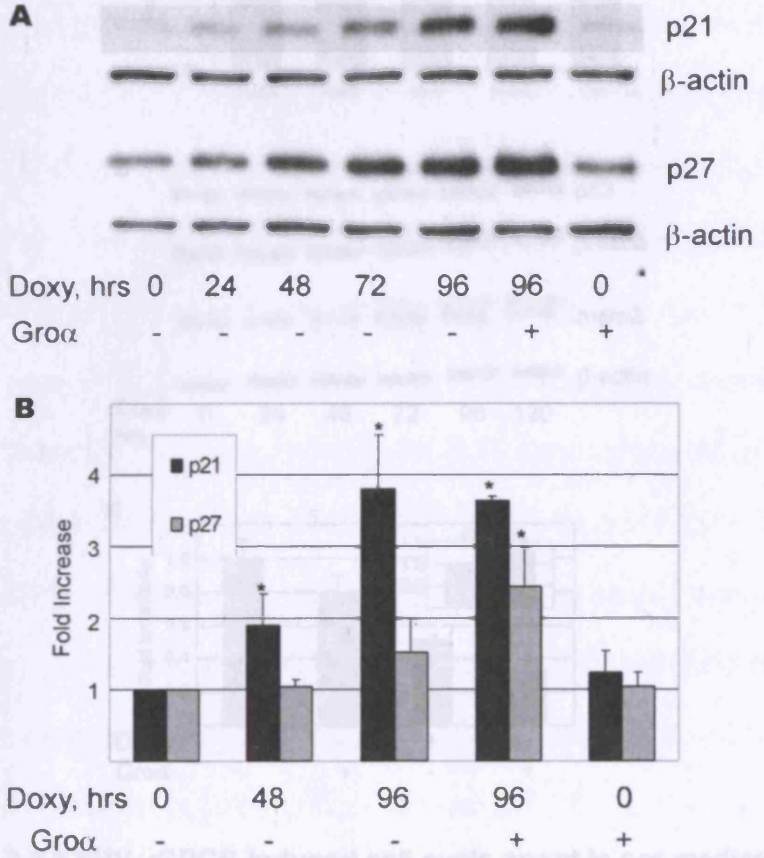


Figure 3.5. KSHV vGPCR upregulates p21 and p27. (**A**) BC3.14 cells were exposed to 2 μg/ml of doxycycline for length of time shown in hours. Western blot shows vGPCR-dependent increases in p21 and p27 over 96 hours. Shown is one of three independent experiments. (**B**) Quantitative RT-PCR shows vGPCR-dependent transcriptional regulation of p21 and to a lesser extent, p27. Groα(100 nm)a vGPCR agonist, is included to assure specificity of observed effects. Average of two independent experiments each done in duplicate is shown. * , significant increase over baseline $p \leq 0.05$.

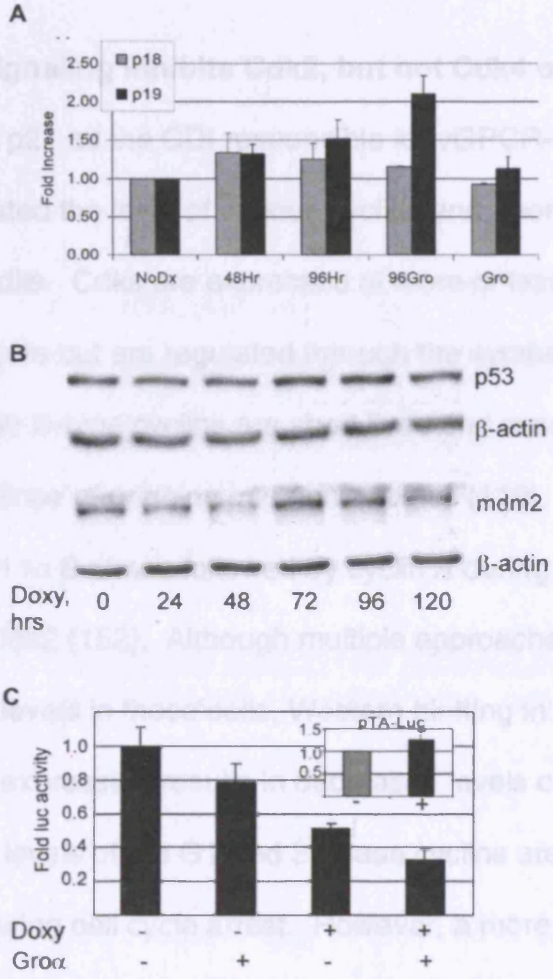


Figure 3.6 KSHV vGPCR-induced cell cycle arrest is not mediated by p18, p19, or p53. (**A**) Quantitative RT-PCR shows lack of vGPCR-dependent transcriptional regulation of p18 and p19 using same cDNA as Fig 3.5. Groα(100 nm) is included to assure specificity of observed effects. Average of two independent experiments each done in duplicate is shown. (**B**) BC3.14 cells were exposed to doxycycline for the specified length of time to induce vGPCR, after which protein lysates (30 μg) were loaded onto 12% SDS-PAGE gels and probed for p53 and mdm2. Shown is one representative of three independent Western blot experiments. (**C**) BC3.14 cells were transfected with p53-TA-luc, a commercially available p53 reporter construct. Transfected cells were then divided and incubated with doxycycline (2 μg/ml for 48 hours), GROα (100nM), or without additives as shown. Protein lysates were harvested and equal amounts assayed. Cells were divided after transfection, so no further control for transfection efficiency was performed. As expected, vGPCR expression has no

3.4 vGPCR signaling inhibits Cdk2, but not Cdk4 or Cdk6 activity

Having identified p21 as the CDI responsible for vGPCR-mediated cell cycle arrest, we next investigated the level of various cyclins and, more importantly, the activity of the relevant Cdks. Cdks are expressed at more or less constant levels throughout the cycle but are regulated through the synthesis and degradation of the cyclins (181). The D-type cyclins are short lived and assemble with Cdk4 or Cdk6 in G1 in the presence of ongoing mitogenic stimuli (119). Cyclin E peaks at the transition from G1 to S phase followed by cyclin A during S phase. Both these cyclins activate Cdk2 (152). Although multiple approaches failed to accurately quantify cyclin E levels in these cells, Western blotting in Figure 3.7A shows that ongoing vGPCR expression results in decreased levels of cyclin A and cyclin D2. This decrease in levels of the G1 and S phase cyclins are consistent with the data in Figure 3.3 showing cell cycle arrest. However, a more definitive functional assay of the cyclin-dependent kinases was performed (148). Using the C-terminus of retinoblastoma protein (Rb) as a substrate, we assayed the kinase activity of Cdk2, 4 and 6 (Figure 3.7B). Although vGPCR had no effect on Cdk 4 or 6, it produced a marked reduction in Cdk2 activity. This is in keeping with the known inhibitory effects of p21 on Cdk2. The effect of p21 on Cdk4/6 is more complex with some models showing inhibition but others arguing that the Cdk/cyclinD interaction with p21 is also important to sequester p21 away from the Cdk2/cyclin E complex (245).

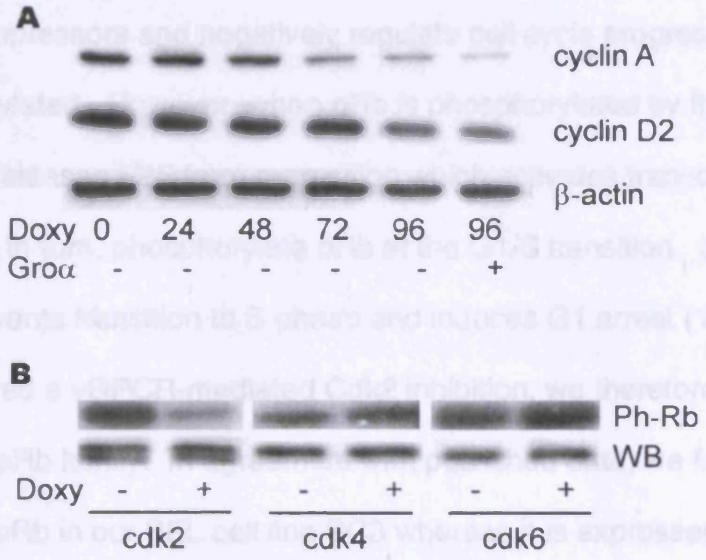


Figure 3.7. KSHV vGPCR causes decreased levels of cyclin A, D2 complexes and decreased cdk2 kinase activity. (**A**) To express vGPCR, BC3.14 cells were exposed to 2 μg/ml doxycycline for the time shown in hours. Protein lysates were run on 10% SDS-PAGE gels and probed with appropriate antibodies. (**B**) BC3.14 cells were incubated for 48 hours with doxycycline and then lysed in kinase buffer. Antibodies were then used to immunoprecipitate each kinase as indicated. After resuspension in kinase buffer, the substrate GST-pRb (C term) was added along with [γ-^{32}P] ATP. Phosphorylated product was detected by autoradiography after separation on a 12% SDS-PAGE gel (top row). 10% of the protein input for the IP was used to Western blot for each kinase (bottom row). One representative of three independent experiments is shown.

The retinoblastoma family of proteins includes pRb, p107 and p130 (for review, see (108)). The phosphorylation status of these proteins is moderated during cell cycle as well as at the cell cycle entry and exit transitions (33, 56). They are tumour suppressors and negatively regulate cell cycle progression when hypophosphorylated. However, when pRb is phosphorylated by the cyclin-D/cdk complex, this releases E2F from repression which activates transcription of cyclins E and A which in turn, phosphorylate pRb at the G1/S transition. Inhibition of cyclin E or Cdk2 prevents transition to S-phase and induces G1 arrest (199, 205). Since we had observed a vGPCR-mediated Cdk2 inhibition, we therefore assessed its effects on the pRb family. In agreement with published data, we found no expression of pRb in our PEL cell line BC3 whereas it is expressed in various other PEL lines (Fig. 3.8A). However, p130 and p107 are also able to mediate cell cycle arrest via interactions with E2F and the cyclins, so we performed Western blots to ascertain whether phosphorylation status or total protein levels varied consistently due to vGPCR expression. Unlike pRb, the levels of p130 and p107 vary with the cell cycle. When cells are forced to exit the cell cycle, p130 levels rise sharply coinciding with accumulation of p130/E2F complexes (134, 172). Although p130 did not vary as detected by Western blots, p107 levels generally seemed to increase with vGPCR-mediated arrest, but the results were inconsistent and we could make no firm conclusions (Fig. 3.8B). Likewise, phosphorylation status as judged by band shifts was not eviedent (data not shown).

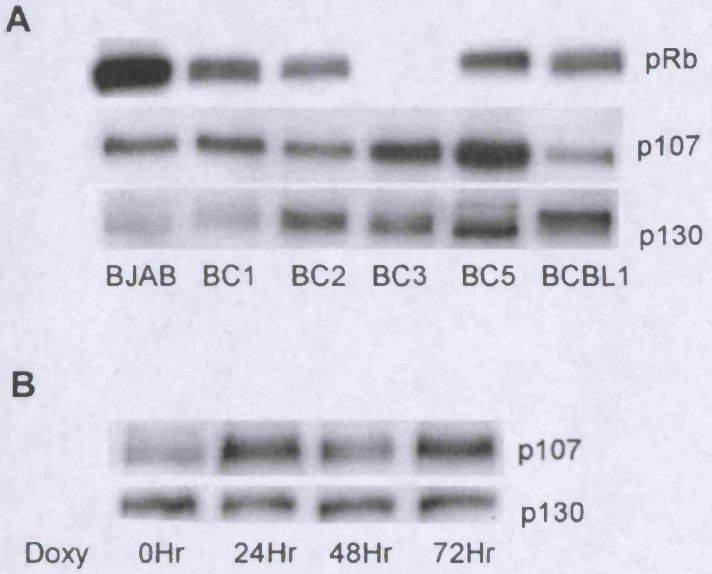


Figure 3.8. KSHV vGPCR has no consistent effect on levels of the pocket proteins p130 and p107. (**A**) Protein lysates of several PEL lines were used in Western blotting to assess protein levels of the pocket proteins pRb, p130, and p107. (**B**) BC3.14 cells exposed to doxycycline as shown were subjected to Western blotting for p130 and p107.

CHAPTER 4:

4.0 Introduction

Herpesviruses manipulate and rely on certain host cell cycle functions for persistence and replication. Unlike small DNA tumour viruses, herpesviruses encode their own DNA polymerase and generally prevent progression to S-phase during replication. However, certain viral products activate some cell cycle regulatory pathways that would normally promote cell cycle progression and inhibition of these pathways inhibits viral replication. There exists, therefore, a general theme that herpesviruses have evolved to block certain cell cycle functions while relying on others. For example HSV-1 IE gene expression requires Cdk activity (128). However, HSV-1 also can mediate a G1 cell cycle arrest to block host cell DNA synthesis (83). Like HSV, CMV can effect a cell cycle arrest and does so via at least two distinct viral products, the tegument protein UL69 and the immediate-early gene product IE2 (282). However, CMV infection also promotes cyclin E levels and cyclin E-associated kinase activity that appears to be required for replication (30). The induction of EBV replication also arrests cell cycle progression in spite of elevated S-phase cdk activity and consequent hyperphosphorylation of pRb (29, 143, 144).

My results in Chapter 3 showing that vGPCR induces a cell cycle arrest could possibly indicate a change from latent to lytic viral life cycle. After all, vGPCR is an IE gene and it is therefore well positioned transcriptionally to affect the latent-to-lytic switch. However, we also found that vGPCR inhibits Cdk2 and as

mentioned above, Cdk2 is necessary for EBV and CMV replication. We therefore wanted to assess vGPCR's effect on the chemical induction of the latent-to-lytic switch in our PEL cell line BC3.14.

CHAPTER 4: RESULTS

4.1 KSHV vGPCR inhibits chemically-mediated lytic gene induction

Having shown that vGPCR overexpression inhibits Cdk2, we used qRT-PCR to quantitate the transcription of KSHV ORF 50 and ORF 26 in chemically induced BC3.14 cells in the presence or absence of vGPCR expression. ORF 50 is an IE transactivator gene of the γ-herpesviruses that is necessary and sufficient to drive lytic replication in KSHV, Rhesus rhadiovirus, and murid herpesvirus-68. Deletion or inhibition by a dominant-negative mutant will prevent lytic replication. ORF 26 is a late lytic gene that encodes a capsid protein. Measuring the induction of these lytic genes is a surrogate for successful lytic replication. Both butyrate and TPA induce PEL lines to produce new virions, although levels of class II and III transcripts vary depending on the stimulus (296). Using standard doses of butyrate and TPA we found that induction of both ORF 50 and ORF 26 transcripts was markedly reduced in BC3.14 cells that were made to express vGPCR prior to induction (Figure 4.1). The effect was most dramatic with TPA and high dose butyrate. Since vGPCR is itself a lytic gene and chemically inducible, we compared the levels of vGPCR transcript achieved with doxycycline induction versus chemical induction. We felt it important to do so since vast overexpression of exogenous signaling proteins can

force otherwise unnatural associations and downstream effects. We found that at 48 to 96 hours of doxycycline induction, levels of vGPCR transcript were comparable to those induced by standard TPA and butyrate doses at 48 hours; doses known to bring about successful virion production (Figure 4.2). Although we have never seen a phenotypic effect of doxycycline on control cell lines (i.e. BC3), we also performed control experiments on BC3 to ensure that doxycycline was not having an unexpected effect on the chemical induction of ORF 50 or ORF 26. Indeed, doxycycline had no independent inhibitory effect on ORF 50 or ORF 26 when assayed by qRT-PCR; curiously however, there was a trend of higher butyrate-induced levels in the presence of doxycycline (Figure 4.2B). The reason for this is unclear but does not detract from the data presented in Figure 4.1.

Since ORF 50 is essential to the KSHV latent-to-lytic switch, it seemed likely that inhibition by vGPCR reflected a global inhibition of lytic gene transcription. However, a preferential inhibition of only certain lytic genes could lead to an 'abortive' or incomplete lytic program. To assess whether vGPCR-mediated inhibition of the chemical induction of ORF 50 and ORF 26 was specific to these lytic genes, primers for qRT-PCR were designed and optimized for the lytic ORFs K8 and K8.1. ORF K8.1 is a late transcript encoding a structural glycoprotein, while K8 is an IE transcript that encodes a basic region-leucine zipper protein of 237 aa that homodimerizes; K8 shows significant similarity to the EBV immediate-early protein Zta. Figure 4.3 shows that vGPCR expression prior to chemical induction also prevented the transcription of K8 and K8.1. Although all lytic genes have not

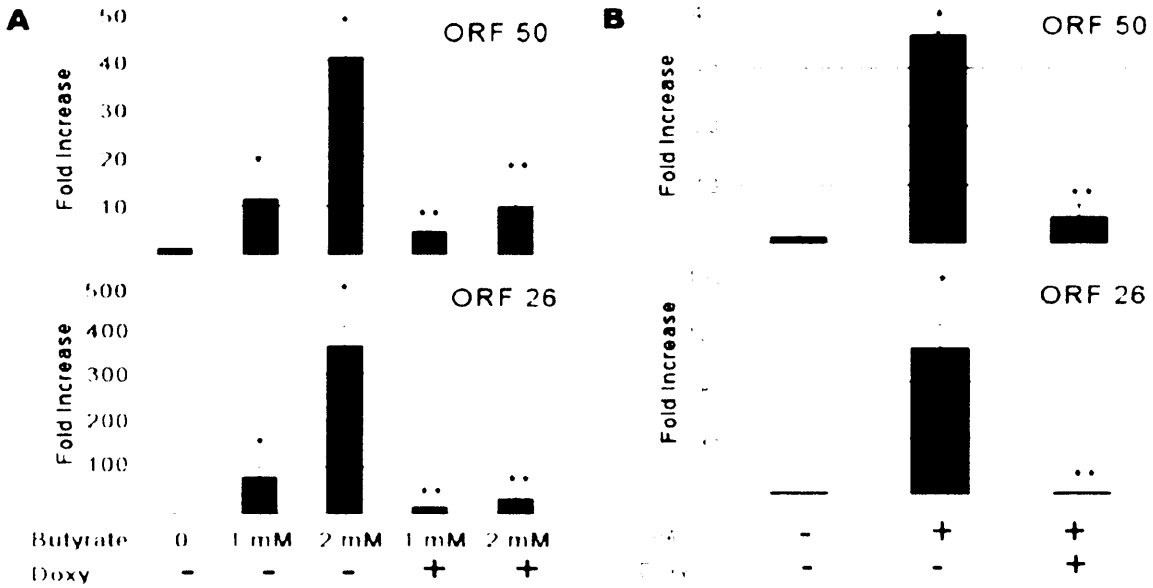
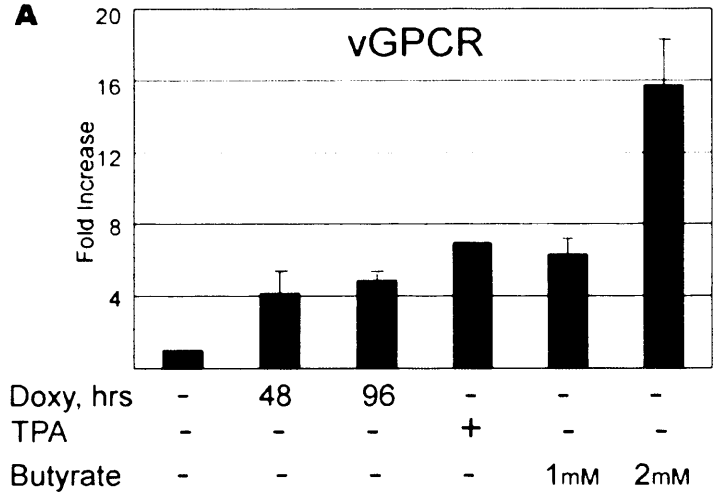


Figure 4.1. KSHV vGPCR expression inhibits lytic gene induction by TPA and butyrate. BC3.14 cells were treated for 48 hours with 2 μg/ml of doxycycline where indicated to express vGPCR, after which cells were also exposed to butyrate (1 mM or 2 mM) (**A**) or TPA (20 ng/ml)(**B**) to induce KSHV lytic gene transcription. qRT-PCR was performed for ORF 50 and ORF 26 message. Results are normalized to untreated cells (lane 1). Shown in both (A) and (B) are two independent experiments each done in duplicate. * , significant increase over baseline $p \leq 0.05$; **, significant decrease from maximum induction, $p \leq 0.05$.

A



B

	Fold Increase in mRNA	
	Orf 50	*Orf 26*
Butyrate	42.8	400.2
TPA	12.6	100.1
Doxy	1.1	0.8
Butyrate, Doxy	85.4	812.3
TPA, Doxy	17.4	125.1

Figure 4.2. Doxycycline induction of KSHV vGPCR in BC3.14 cells results in increases in vGPCR message similar to levels obtained with lysis-inducing chemicals. (**A**) BC3.14 cells were treated for 48 hours with TPA (20 ng/ml), butyrate (1 mM and 2 mM), or 2 μg/ml of doxycycline (48 and 96 hours). Quantitative RT-PCR was then done using primers for KSHV vGPCR. Results were normalized to untreated cells in lane 1. (**B**) Table summarizes quantitative RT-PCR results for ORF 50 and ORF 26 in BC3 cells treated for 48 hours with butyrate (2 mM), TPA (20 ng/ml), doxycycline (2 μg/ml) or combinations as shown. Results shown are average of two independent experiments and indicate that doxycycline has no independent negative effect on induction of ORF 50 or ORF 26.

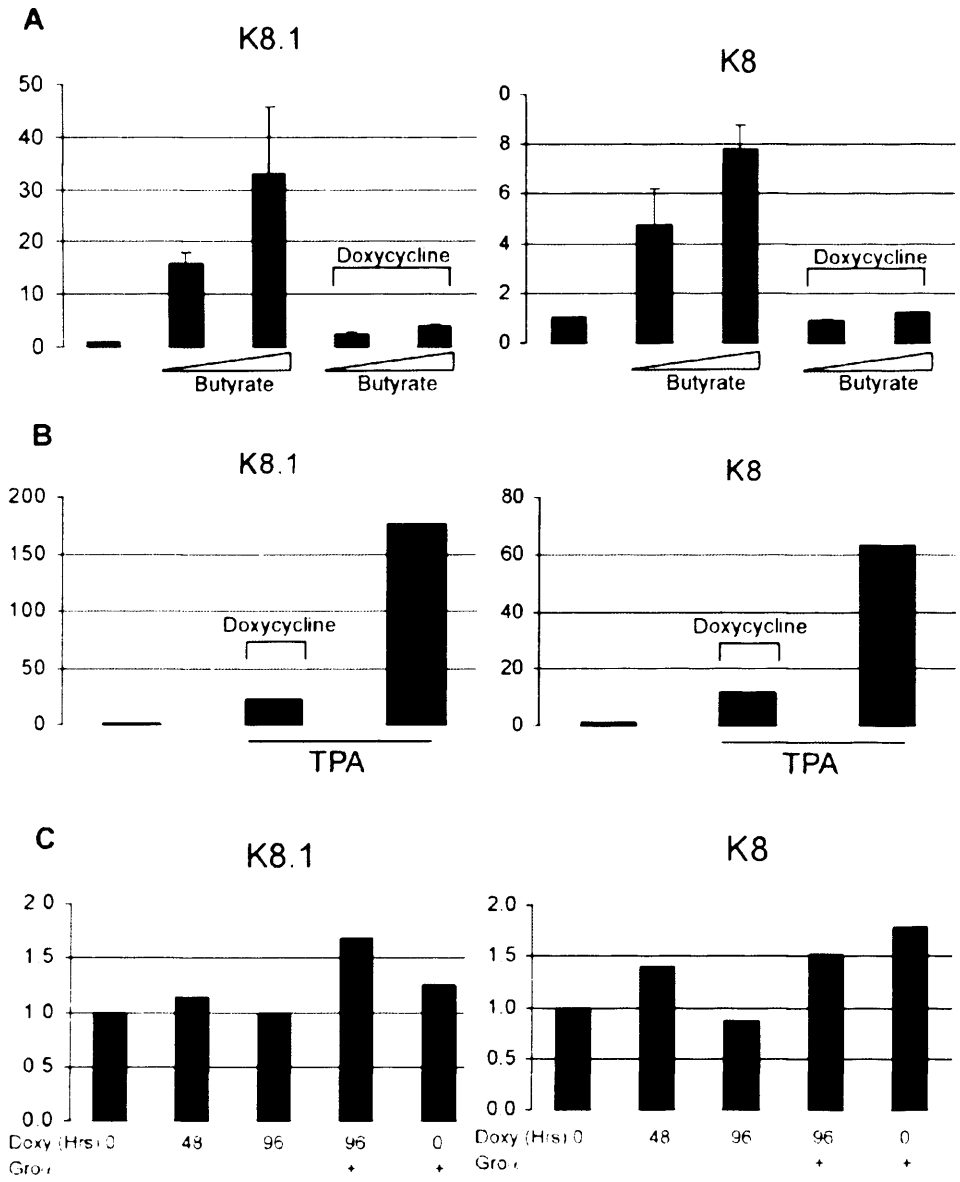


Figure 4.3. vGPCR expression prevents chemical induction of KSHV lytic genes K8.1 and K8. BC3.14 cells were treated for 48 hours with 2 μg/ml of doxycycline where indicated to express vGPCR, after which cells were also exposed to butyrate (1 mM or 2 mM) (**A**) or TPA (20 ng/ml)(**B**) to induce KSHV lytic gene transcription. qRT-PCR was performed for K8.1 and K8 message. Results are normalized to untreated cells (lane 1). Shown in both (**A**) are two independent experiments each done in duplicate. (**B**) and (**C**) were performed once in duplicate and given negative results were not repeated.

been tested, these results strongly suggest that vGPCR has a global inhibitory effect on KSHV lytic gene transcription.

4.2 KSHV vGPCR inhibits lytic activation via inhibition of Cdk2

As discussed, Cdk2 activity is necessary for both EBV and CMV replication. We therefore assessed specifically whether the vGPCR-induced inactivation of Cdk2 could be at least partially responsible for the decrease in chemically induced transcription of ORF 50 and 26. Using lentiviral transduction, we infected BC3.14 cells with either a Cdk2 dominant negative (DN) construct or a reverse Cdk2-DN sequence as a control (272). Cells were incubated with butyrate; ORF 50 and 26 transcripts were quantified by qRT-PCR. Figure 4.4 shows that the Cdk2 dominant negative caused an approximately 50% reduction in both transcripts. Of note, these data allow us to conclude that it is the vGPCR-induced inhibition of Cdk2 that results in inhibition of ORF50 and ORF26 transcripts, strongly suggesting an impairment of successful lytic replication.

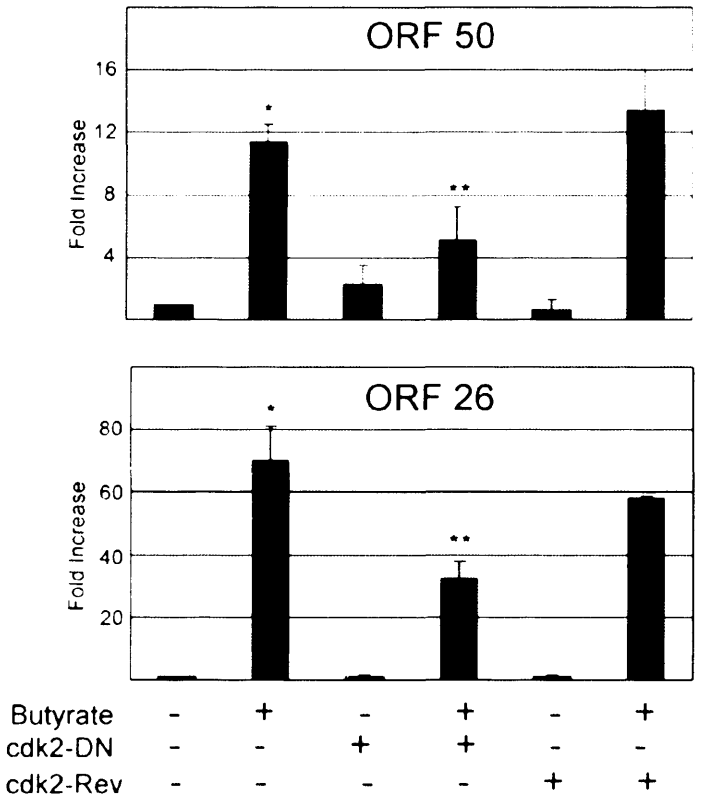


Figure 4.4. Cdk2 activity is necessary for full chemical induction of KSHV lytic genes. BC3 cells were infected with lentivirus expressing the Cdk2 dominant negative coding sequence in either forward or reverse (control) orientation. 72 hours later, cells were treated with 1 mM butyrate for 48 hours as indicated. Quantitative RT-PCR was performed for ORF 50 (top panel), and ORF 26 (lower panel). Figure represents two independent experiments each done in duplicate. * , significant increase over baseline $p \leq 0.05$; **, significant decrease from maximum induction, $p \leq 0.05$.

CHAPTER 5:

5.0 Introduction

KSHV vGPCR is known to signal broadly via multiple Gα subtypes and via several growth-related tyrosine kinase pathways. However, nothing is yet known about the vGPCR's effects on the protein tyrosine phosphatases (PTPs) which are just as crucial to extracellular signaling as PTKs and are increasingly the target of pharmacologic inhibitor design. As discussed in Chapter 1.13, the ITIM motif is a consensus sequence that when tyrosine-phosphorylated can bind one or more of the PTPs, thereby causing a conformational change in the PTP and releasing basal auto-inhibition of the phosphatase catalytic domain. A receptor that contains a cytoplasmic ITIM motif is therefore a valid candidate as an inhibitory receptor and an activator of the PTPs. We have identified an ITIM motif at the junction of the seventh transmembrane domain and the cytoplasmic tail of the KSHV vGPCR. Although it is still debatable whether GPCRs directly bind to PTPs generally, there is one report that the bradykinin B2 receptor binds SHP-2 via an ITIM at the proximal cytoplasmic tail (LVYVIV) (71). Bradykinin stimulation transiently increases the association of this GPCR with SHP-2 and enhances its phosphatase activity. Although SHP-2 is generally thought to play a positive role in growth factor signaling, the authors show that SHP-2 activation is required for the anti-mitogenic effect bradykinin has on proliferating renal mesangial cells.

Although the KSHV vGPCR has mitogenic effects in standard transformation assays and enhances the longevity of HUVECS, we have noted that overexpression

of vGPCR is detrimental to some cell types. Although this may be a function of expression levels of vGPCR, it is possible that as in the case of the bradykinin receptor, vGPCR may have both positive and negative effects on cell growth; the cell cycle arrest in PEL cells described in Chapter 3 is an example of the latter. This possibility combined with the presence of an ITIM motif requires that vGPCR be further investigated for a possible association with the PTPs. Although we hypothesize that the vGPCR ITIM facilitates an interaction with one or more of the PTPs, it is possible that such an interaction could occur in an ITIM-independent manner. For example, Feng et al show that despite the presence of an ITIM in the 3rd cytoplasmic loop, the angiotensin II AT_2 receptor activates SHP-1 in an ITIM-independent fashion via a Gαs subunit (81). Furthermore, it must also be kept in mind that the selectivity of various ITIM motifs for the different SH2-containing PTPs is not wholly understood. Therefore, receptor interaction with one SHP subtype cannot be generalized to other subtypes; each must be individually investigated.

CHAPTER 5: RESULTS

5.1 KSHV vGPCR activity phosphorylates SHP-2 at Y542 but not Y580.

SHP-1 and SHP-2 undergo tyrosine phosphorylation of tyrosine residues in their C-terminal tails (see Fig. 5.1). Whether this phosphorylation regulates phosphatase activity or merely enhances the role of these PTPs as scaffolding molecules is controversial. Lorenz et al. saw no change in phosphatase activity upon SHP-1 phosphorylation at Y536 and Y564 in CD4- or CD8-stimulated T cells (162). Moreover, they hypothesized that the phosphorylated tyrosines could bind to the SH2 domains of Grb2, a molecule now known to be recruited by SHP-1 (177). On the other hand, others have shown that phosphorylation of the homologous tyrosines on SHP-2 does indeed increase phosphatase activity (80). Since both SHP-1 and SHP-2 can auto-dephosphorylate, the phosphorylation status of the C-terminal tyrosines is labile and notoriously difficult to study. Elegant work using non-hydrolysable mimics of phosphotyrosine in place of the wild-type residues, shows that SHP-2 phosphatase activity is slightly increased and SHP-1 activity markedly increased by these substitution (80, 298). There is no thorough mechanistic investigation of how phosphorylation of C-terminal tyrosines could induce phosphatase activity. However, the above authors hypothesize that the phosphotyrosyl residues bind intramolecularly to the SH2 sites thereby causing the necessary allosteric switch. Regardless of their exact role, the high degree of evolutionary conservation suggests that the C-terminal phosphotyrosyl residues place an important role in SHP-1/2-mediated signaling.

Given the ubiquitous nature of SHP-2 and the availability of phospho-specific antibodies, we chose to start our investigation of vGPCR-mediated effects on PTPs by evaluating whether vGPCR increases C-terminal tyrosine phosphorylation of SHP-2. Using increasing doses of a vGPCR expression construct, we found that vGPCR enhanced phosphorylation of SHP-2 Y542 in both NIH 3T3 and HEK 293 cells (Fig 5.2). No change in baseline phosphorylation of Y580 was seen. Since many investigators study the PTPs under serum starvation conditions to decrease baseline activation, we did the same; the same vGPCR-induced effect was seen (data not shown). For further studies we used HEK 293 for ease of manipulation and transfection.

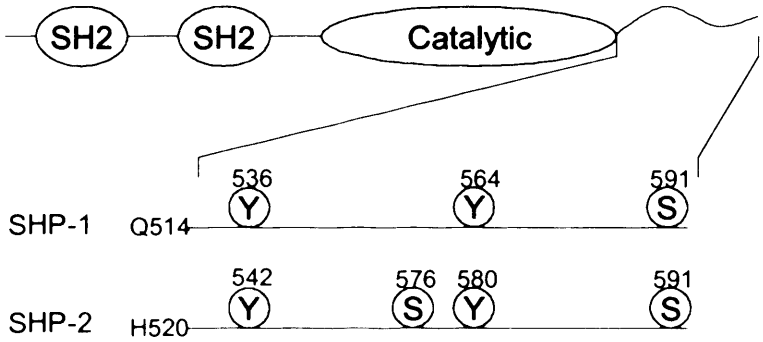


Figure 5.1. Schematic of SHP-1 and SHP-2 C-terminal tails. *Adapted from Jones, M (2005) Cell Sig (17):p1323*

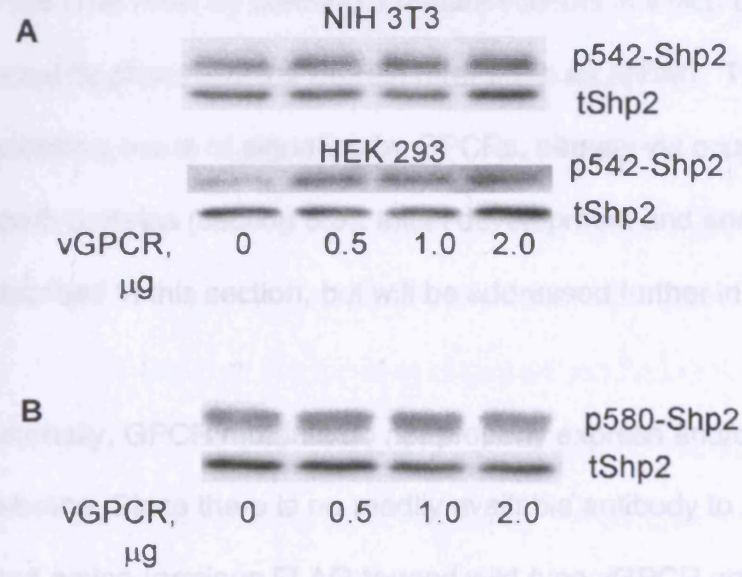


Figure 5.2. KSHV vGPCR expression results in phosphorylation of SHP-2 at Y542, but not Y580. (**A**) NIH 3T3 cells or HEK 293 cells were transfected with increasing amounts of pcKSHV-vGPCR. After 48 hours, cells were lysed in RIPA buffer and Western blotting performed for Y542-phosphorylated SHP-2. Blots were then stripped and re-probed for total SHP-2. (**B**) HEK 293 lysates were also probed for Y580 phosphorylation. Shown are representative blots of three independent experiments.

5.2 The vGPCR Y142F ITIM mutant properly expresses and localizes to the plasma membrane

To delineate the signaling events responsible for vGPCR-mediated SHP-2 phosphorylation, we took two simultaneous directions. One was to evaluate the necessity of the ITIM motif by creating a mutant vGPCR in which the tyrosine of the ITIM is replaced by phenylalanine (herein referred to as ΔITIM). The other was to assess the classical mode of signaling by GPCRs, namely via coupling to heterotrimeric G proteins (section 5.3). Initial development and analysis of the ΔITIM mutant is described in this section, but will be addressed further in sections 5.4 and 5.5.

Occasionally, GPCR mutants do not properly express and/or localize to the plasma membrane. Since there is no readily available antibody to KSHV vGPCR, we engineered amino-terminus FLAG-tagged wild-type vGPCR and ΔITIM constructs. As shown by immunofluorescence in Figure 5.3A, the mutant receptor properly localizes to the plasma membrane. Flow cytometry (FCM) shows that the mean fluorescence (MF) of cells transfected with equal amounts of wild-type and mutant vGPCR are equal, confirming that the ΔITIM mutant is expressed as efficiently as the wild-type.

As a functional assay of the newly designed ΔITIM mutant, it was compared to wild-type vGPCR in its ability to stimulate NFκB-mediated transcription of a luciferase reporter construct (Fig. 5.3B). Despite equal expression, the ΔITIM mutant activated NFκB to approximately 50% of the wild-type. Of note, the amino-terminal FLAG epitope tag does not appreciably interfere with constitutive NFκB

activation by the wild-type or ΔITIM vGPCR. Binding studies to assess interference with ligand binding to the extracellular amino terminus were not done since the FLAG-tagged constructs were used for IP studies in the absence of any exogenous ligand. Further characterization of the ΔITIM mutant will be shown in later figures.

5.3 vGPCR phosphorylates SHP-2 via a Gαq-Src axis

Although the ITIM domain could possibly be involved with the vGPCR-mediated phosphorylation of SHP-2, we also performed studies to determine if the more classical G protein activation by the vGPCR could be responsible. vGPCR is known to couple to both Gαi and Gαq subtypes of G protein. In Figure 5.4A, pertussis toxin (200 ng/ml), a pharmacologic inhibitor of Gαi subunits was added to cell culture 12-17 hours prior to making protein lysates. These experiments show that Gαi subunits are not necessary to either baseline or vGPCR-induced SHP-2 phosphorylation. In some studies ERK is shown to be upstream of SHP1/2 activation and in others downstream. We used a pharmacologic inhibitor of ERK, PD98059, in standard doses (25 μm) and found that ERK activity is not required for SHP-2 phosphorylation by vGPCR. As mentioned above, there are reports that Src kinase family members are required to activate SHP1/2. Furthermore, we and others have shown that vGPCR can activate Src family members (37). Therefore, PP2 (5 μm), a specific pharmacologic inhibitor of Src, was also assessed for its effect. Src inhibition resulted in dramatic inhibition of both baseline SHP-2 Y542 phosphorylation and vGPCR-induced phosphorylation. To verify the PP2 data, a commercially available dominant-negative construct of Src was also evaluated and

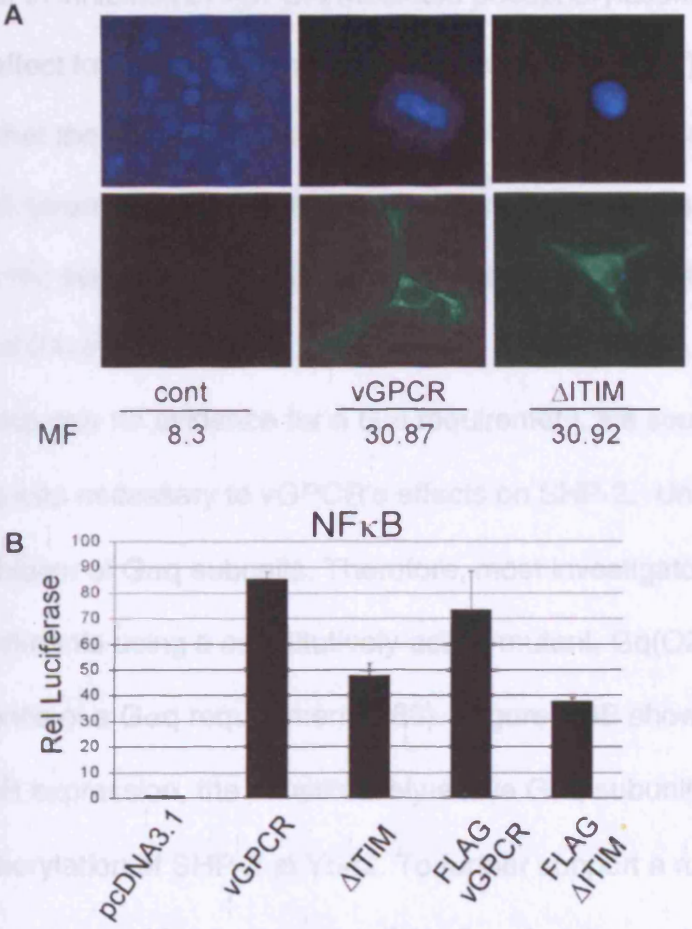


Figure 5.3. The vGPCR-ΔITIM mutant properly localizes to cell membrane and is expressed at similar levels as wild-type vGPCR, but is less efficient at activating NFκB. (**A**) HEK 293 cells were transfected with equal amounts of plasmid expressing either FLAG-vGPCR or the FLAG-vGPCR ΔITIM mutant. After 48 hours, cells were stained with anti-FLAG antibody and DAPI to stain nuclei. Cells were also evaluated by FCM and mean fluorescence (MF) was very similar for wt and mutant vGPCR. (**B**) HEK 293 cells were transfected with constructs as indicated and NFκB-mediated expression of luciferase measured at 48 hours post-transfection. Bars indicate S.D. of three independent experiments.

found to result in inhibition of vGPCR-mediated phosphorylation, with no appreciable effect found on baseline phosphorylation (Fig. 5.4C). This is not surprising in that the dominant-negative construct would not be expected to be as potent as PP2 given issues of transfection efficiency and expression of the former. Nonetheless, the combined PP2 and dominant-negative data strongly support the involvement of Src in this pathway.

Since we saw no evidence for a Gαi requirement, we sought to determine if Gαq coupling was necessary to vGPCR's effects on SHP-2. Unfortunately there is no reliable inhibitor of Gαq subunits. Therefore, most investigators rely on gain-of-function experiments using a constitutively-active mutant, Gq(Q209L), to obtain indirect evidence of a Gαq requirement (288). Figure 5.4B shows that despite the lack of vGPCR expression, the constitutively-active Gαq subunit is sufficient to cause phosphorylation of SHP-2 at Y542. To further support a role for Gαq as opposed to Gαi, a possible requirement of the βγ subunit was also evaluated. Although both Gαi and Gαq G proteins can signal via their βγ subunits, Gαi is more dependent on the βγ subunit and the α subunit has weak signaling potential of its own. A commonly used approach to inhibit βγ signaling is to co-express the G protein α subunit of retinal transducin (αT) as a scavenger of βγ subunits (79). Figure 5.5A shows that αT has no effect on SHP-2 phosphorylation. As a positive control to show that the αT subunit can be expressed and Gαi coupling is necessary to certain vGPCR functions in this cell type, an NFκB reporter assay was

performed in 5.5B. By scavenging $\beta\gamma$ subunits, vGPCR-mediated NFκB activation was decreased by approximately 60%.

5.4 The vGPCR does not activate SHP-2 phosphatase activity and the ITIM motif is not required for SHP-2 phosphorylation

The data in Figures 5.4 and 5.5 and the fact that vGPCR activates Src, argues for a vGPCR-Gαq-Src axis as responsible for SHP-2 phosphorylation in our model. Since expression of the constitutively active Gq(Q209L) results in the phosphorylation of SHP-2 in the absence of vGPCR, it would be hard to argue that the vGPCR ITIM motif is necessary. Moreover, given that interaction with an ITIM motif activates the PTPs, it would be expected that any direct interaction of SHP-2 with the vGPCR ITIM would enhance SHP-2 phosphatase activity. We therefore assessed this activity by immunoprecipitating SHP-2 after transfection with vGPCR, mutant vGPCRs, or Gq(Q209L). Immunoprecipitated enzyme was incubated with a phosphorylated peptide and free phosphate release measured. As shown in Figure 5.6, neither vGPCR, the ΔITIM mutant or the constitutively-active Gαq subunit activated SHP-2 phosphatase activity. Of note, we also included a vGPCR mutant in which the VRY motif in the second cytoloop was changed to ALY (ΔALY). This VRY motif is a variant of the very well conserved DRY motif which is crucial to G protein coupling with GPCRs.

Together, the data presented in Figures 5.5 and 5.6 support a signaling pathway as shown in Figure 5.7. Despite a lack of phosphatase activity, the vGPCR-induced SHP-2 phosphorylation may well be required for promoting SHP-2 scaffolding functions. This possibility will be discussed in Chapter 7.

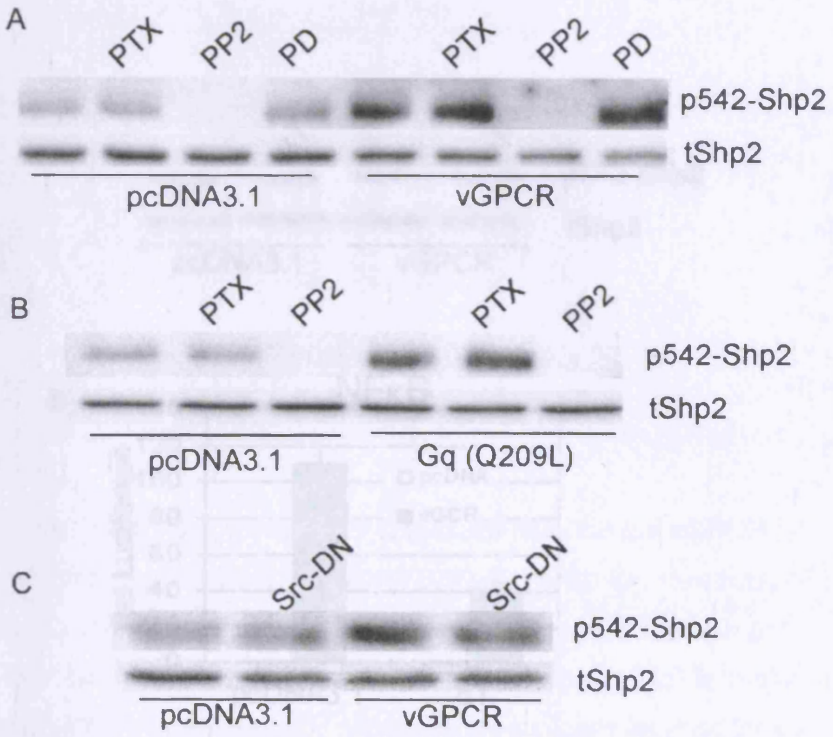


Figure 5.4. vGPCR-induced SHP-2 phosphorylation is Gαq- and Src-dependent but ERK independent. HEK 293 cells were transfected with either control plasmid, vGPCR (**A** and **C**), or the constitutively active Gαq mutant Gq(Q209L) (**B**). After 36 hours, inhibitors were added where shown (A and B). In panel (**C**), Src dominant negative construct was co-transfected as shown. Protein lysates were made 48 hours post-transfection and Western blot performed for Y542-phosphorylated SHP-2. Blots were stripped and re-probed for total SHP-2. PTX, pertussis toxin (200μg/ml), PP2 (5 μM), PD, PD98059 (25 μM).

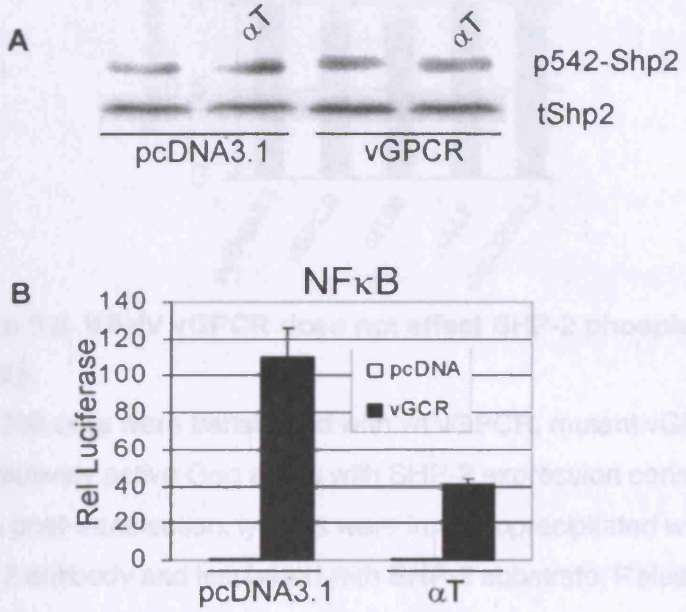


Figure 5.5. KSHV vGPCR-mediated phosphorylation of SHP-2 is not dependent on βγ subunits. (**A**) HEK 293 cells were transfected with vGPCR or control plasmid as shown with or without a-transducin subunit. 48 hours post-transfection, protein lysates were subjected to Western blotting for Y542-phosphorylated SHP-2. Blots were then stripped and re-probed for total SHP-2. (**B**) HEK 293 cells were co-transfected with NFκB-luciferase reporter construct and either vGPCR or control plasmid; α-transducin subunit was also transfected where indicated. 48 hours post-transfection, lysates were harvested and luciferase activity determined. All samples were also transfected with TK-Renilla construct to control for transfection efficiency. Values are normalized to control point. Bars represent S.D.

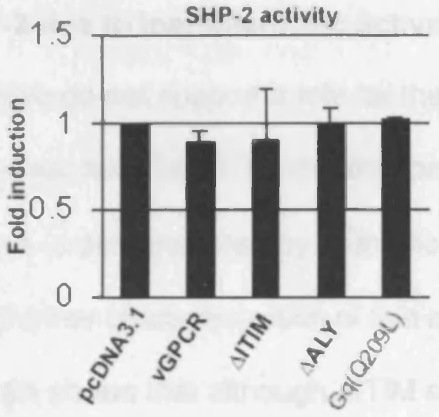


Figure 5.6. KSHV vGPCR does not affect SHP-2 phosphatase activity.

HEK 293 cells were transfected with wt vGPCR, mutant vGPCR, or constitutively active Gαq along with SHP-2 expression construct. 48 hours post-transfection, lysates were immunoprecipitated with anti-SHP-2 antibody and incubated with SHP-2 substrate. Release of free phosphate was measured by malachite green assay as described in Materials and Methods. Results were normalized to control point

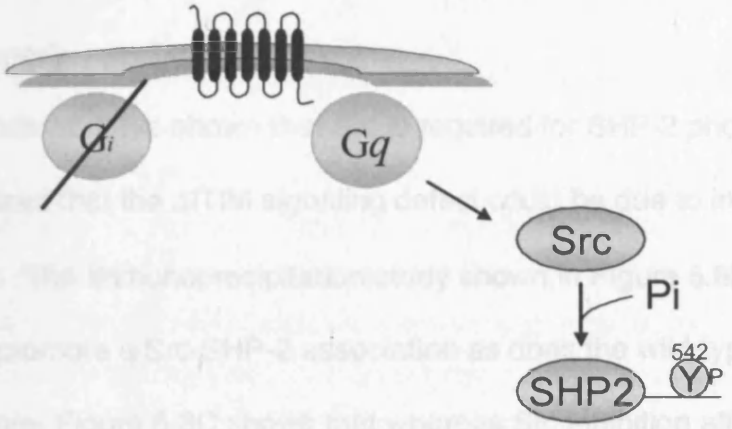


Figure 5.7 Schematic of vGPCR-mediated SHP2 phosphorylation

5.5 The vGPCR mutant ΔITIM is unable to fully activate ERK or to phosphorylate SHP-2 due to inefficient Src activation

Although the data above do not support a role for the vGPCR ITIM in SHP-2 phosphorylation, it is clear that the ΔITIM mutant does not function as well as the wild-type receptor. This is demonstrated by its inefficient NFκB activation shown in Figure 5.3B. We felt further characterization of this interesting new mutant was warranted. Figure 5.8A shows that although ΔITIM expression results in some increase in SHP-2 phosphorylation, it is less than wild-type vGPCR. As expected this is also the case with the negative control, ΔALY, which cannot properly couple to G proteins. An inability of ΔITIM to fully activate ERK is also evident. Of interest we have also found that vGPCR signaling results in phosphorylation of focal adhesion kinase (FAK). Although the study of vGPCR's effects on FAK and cell morphology is the subject of other ongoing studies, it is not addressed specifically in this thesis; we simply include the data here as further evidence that ΔITIM fails to signal properly.

Since we have shown that Src is required for SHP-2 phosphorylation, we hypothesized that the ΔITIM signaling defect could be due to inefficient Src activation. The immunoprecipitation study shown in Figure 5.8B shows that ΔITIM does not promote a Src-SHP-2 association as does the wild-type vGPCR. Furthermore, Figure 5.8C shows that whereas Src inhibition affects NFκB-mediated transcription by wild-type vGPCR and constitutively-active Gq(Q209L), it has no inhibitory effect on ΔITIM-mediated activation. Although just a pilot experiment currently being further optimized, the Src-kinase assay shown in Figure 5.9

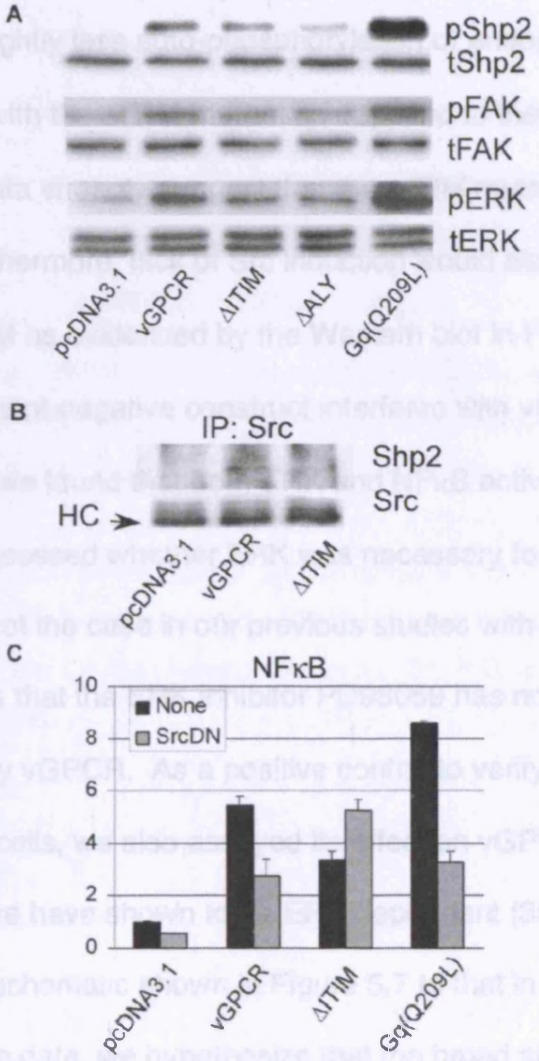


Figure 5.8. The vGPCR-ΔITIM mutant is less efficient at phosphorylating SHP-2 and ERK compared to wt vGPCR due to decreased activation of Src. (**A**)HEK 293 cells were transfected with wt vGPCR, mutant vGPCR or constitutively active Gq(Q209L) as shown. 48 hours after transfection, Western blots for phosphorylated SHP-2, FAK, or ERK were performed as indicated. Blots were then stripped and re-probed for total enzyme levels in each case. Shown is representative of three independent experiments. (**B**) HEK 293 cells were transfected as above and lysates immunoprecipitated with anti-Src antibody, followed by immunoblotting for SHP-2. Blots were stripped and probed for Src to show similar levels of immunoprecipitated protein, HC, heavy chain. (**C**) HEK 293 cells were transfected with an NFκB-luciferase reporter gene construct along with expression constructs as indicated on x-axis, +/- Src dominant-negative construct. Luciferase assays were performed 48 hours post-transfection. Shown is average of two separate experiments done in duplicate. Bars indicate S.D.

seems to show slightly less auto-phosphorylation of endogenous Src in HEK 293 cells transfected with the ΔITIM mutant as opposed to the wild-type vGPCR. Together these data strongly suggest that the ΔITIM mutant is unable to completely activate Src. Furthermore, lack of Src induction would also explain poor ERK activation by ΔITIM as evidenced by the Western blot in Figure 5.10 which shows that the Src dominant-negative construct interferes with vGPCR-induced ERK activation. Since we found that both ERK and NFκB activation by vGPCR require Src activity, we assessed whether ERK was necessary for NFkB activation in these cells. Such was not the case in our previous studies with haematopoietic cells (38). Figure 5.11 shows that the ERK inhibitor PD98059 has no significant effect on NFκB activation by vGPCR. As a positive control to verify that the ERK inhibitor is effective in these cells, we also assayed its effect on vGPCR-mediated activation of AP-1; a process we have shown to be ERK dependent (38). Figures 5.8-5.11 allow us to expand the schematic shown in Figure 5.7 to that in Figure 5.12.

Given these data, we hypothesize that the broad signaling defect of the ΔITIM mutant is due to improper activation of Src, likely via poor G protein coupling. This may be due to conformational changes that the mutation confers. Investigating this hypothesis is beyond the scope of this thesis but will be the subject of future work.

SHP-2 has been associated with signaling via various growth factors and cytokines and with signal transduction processes such as the Ras-Raf-MAPK, JAK-STAT, PI3K, and NFκB pathways. However, other than the EGF receptor, the precise substrates for SHP-2 are not worked out and are so far identified only as

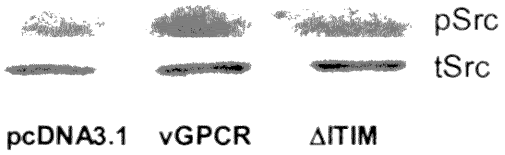


Figure 5.9. ΔITIM mutant is less efficient at Src activation than wild-type vGPCR. HEK 293 cells were transfected with either control plasmid, vGPCR or ΔITIM mutant. 48 hours post-transfection lysates immunoprecipitated with anti-Src antibody. Immuno-complexes were then used in kinase assay in which Src activity is measured by its ability to self-phosphorylate in presence of radio-labeled ATP. Lysates were then run on 10% PAGE gel, fixed, dried, and exposed.

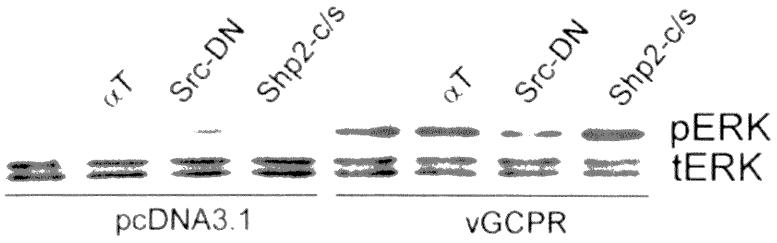


Figure 5.10. SHP-2 is not required for KSHV vGPCR-mediated activation of ERK. HEK 293 cells were co-transfected with either vGPCR or control plasmid along with α-transducin subunit, Src dominant negative or SHP-2 dominant negative as shown. 48 hours post-transfection lysates were subjected to Western blotting with anti-phospho-ERK antibody. Blots were then stripped and re-probed for total ERK. Blot shown is representative of three independent experiments.

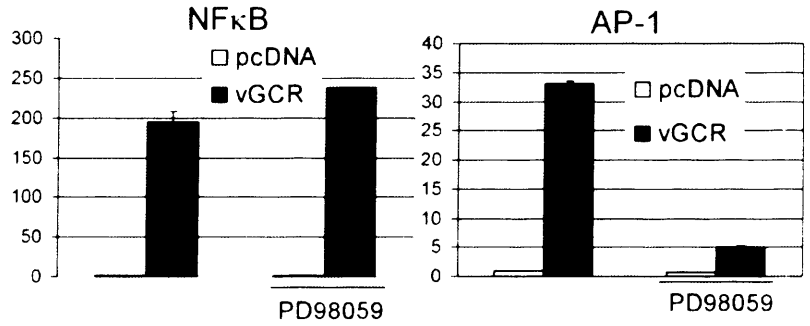


Figure 5.11 vGPCR activation of NFκB is not ERK dependent. HEK 293 cells were transfected with control plasmid or vGPCR as shown. In addition NFκB (left panel) or AP-1(right panel) luciferase reporter constructs were also transfected. All samples included TK-Renilla expression plasmid to control for transfection efficiency. 36 hours after transfection, the ERK inhibitor PD98059 (25 μm) was added where indicated. Cells were lysed after 48 hours and Luciferase assays performed. Results are normalized to control. Shown is average of two independent experiments. Bars indicate S.D.

p90, p120, and p150 (173). Given that vGPCR activates both the MAP kinase and NFκB pathways, we used a SHP-2 dominant-negative substrate-trapping construct, SHP-2(c/s) (gift of Yehenew Agazie), to determine whether SHP-2 is necessary to either of these pathways. Figure 5.10 shows that SHP-2(c/s) did not inihibit vGPCR-mediated ERK phosphorylation. Furthermore, the luciferase reporter assays in Figure 5.13 show that SHP-2(c/s) actually enhanced both vGPCR-mediated NFκB and AP-1 activity. The cause for this increase is not clear. It is, however, not unexpected that inhibition by SHP-2(c/s) was not seen given our earlier experiments showing no vGPCR-induced increase in SHP-2 phosphatase activity. Moreover, these experiments cannot completely rule out a minor contribution of SHP-2 to vGPCR-mediated ERK activation, but do indicate that other vGPCR-induced events are either more potent or are recruited to activate ERK in the absence of SHP-2.

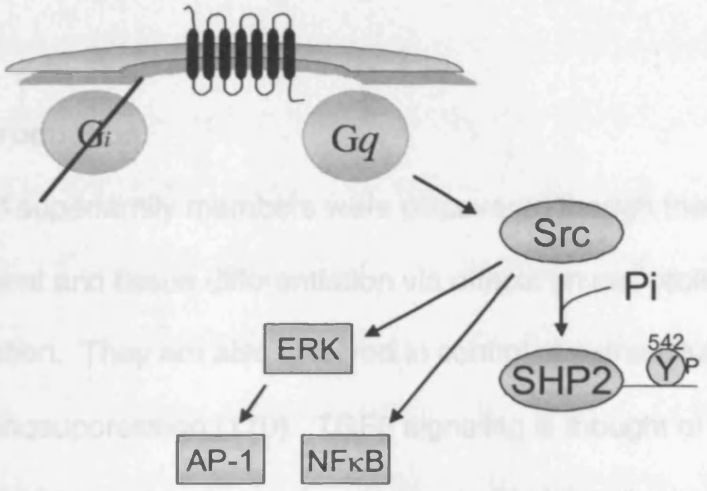


Figure 5.12. Expanded schematic of vGPCR activation of Src, SHP-2, ERK, and the transcription factors AP-1 and NFκB.

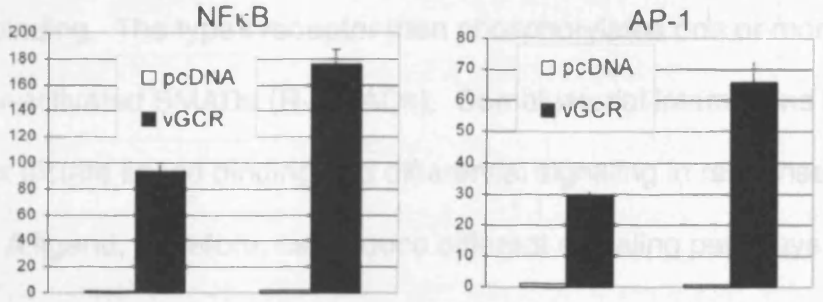


Figure 5.13. SHP-2 c/s, a dominant-negative mutant of SHP-2, does not interfere with vGPCR-induced NFkB or AP-1 activation. BC3.14 cells were transfected with the appropriate luciferase reporter construct with and without SHP-2 c/s. 48 hours post transfection cells were lysed and lucuferase assays performed. Shown is average of two independent experiments. Bars represent S.D.

CHAPTER 6

6.0 Introduction

The TGFβ superfamily members were discovered though their role in body plan development and tissue differentiation via effects on cell proliferation, differentiation and migration. They are also involved in control of extracellular matrix formation and immunosuppression (170). TGFβ signaling is thought of as a linear pathway from the TGFβ type I and type II receptors to SMAD activation, which results in the ligand-induced transcription associated with TGFβ (126). The functional complex of the TGFβ receptors consists of two type I and two type II transmembrane serine/threonine kinases receptors. Type I receptors have a Gly-Ser sequence upstream of the kinase domain that is phosphorylated by the type II receptor upon ligand binding. The type I receptor then phosphorylates one or more of the receptor-activated SMADs (R-SMADs). Combinatorial interactions in the receptor complex dictate ligand binding and differential signaling in response to a given ligand. A ligand, therefore, can induce different signaling pathways depending on the composition of the receptor complex (68). For example, TβRII can interact with TβRI resulting in activation of SMAD2 and SMAD3; when TβRII binds to ALK1, however, SMAD1 and SMAD5 are activated. Sometimes such differential signaling occurs within the same cells, making it very difficult to determine the overall effects of TGFβ (107).

Once bound to ligand, the TβRI receptor phosphorylates the carboxy-terminus of the R-SMADs such as SMAD2 and SMAD3, which are released from

the receptor complex to form a heterotrimeric complex comprising two R-SMADs and a common SMAD4. SMAD4 contains both nuclear localization signals and nuclear export signals which result in constant shuttling between cytoplasm and nucleus. The R-SMADs however, localize to the nucleus only after activation and association with SMAD4 (see Figure 6.1) (for review, see (69)). TGFβ-mediated transcription is determined by the physical interaction and functional cooperation of the SMADs with sequence-specific transcription factors. SMAD affinity to DNA in itself is weak, but it is required for transcriptional activation. In addition to a large array of DNA-binding transcription factors, the R-SMADs interact with co-activators such as CBP/p300 as well as co-repressors. SMAD4 helps to stabilize the interaction of the R-SMADs with DNA and CBP/p300 (124).

TGFβ generally has an inhibitory effect on cell growth; for example it inhibits c-myc and can activate the cyclin-dependent kinase inhibitors, thus stalling cell cycle progression (54). It can also inhibit differentiation in myoblasts, osteoblasts and adipocytes. Given its cytostatic effects, it is not surprising that dysregulation of the TGFβ pathway has been noted in several malignancies: breast cancer, T cell lymphomas, colon cancer, and head and neck squamous cell cancer to name a few (95, 114, 130, 167, 262). Haematologic malignancies are associated with several different mechanisms of TGFβ pathway interference at the receptor and SMAD levels. As mentioned in section 1.14, HTLV-1 Tax inhibits SMAD3/4 formation and SMAD3 DNA binding (182). In the case of chronic myeloid leukemia, EVI-1, a zinc-finger protooncogene, is overexpressed. The first zinc-finger domain of EVI-1 associates with Smad3 and represses its transcriptional activity by recruiting histone

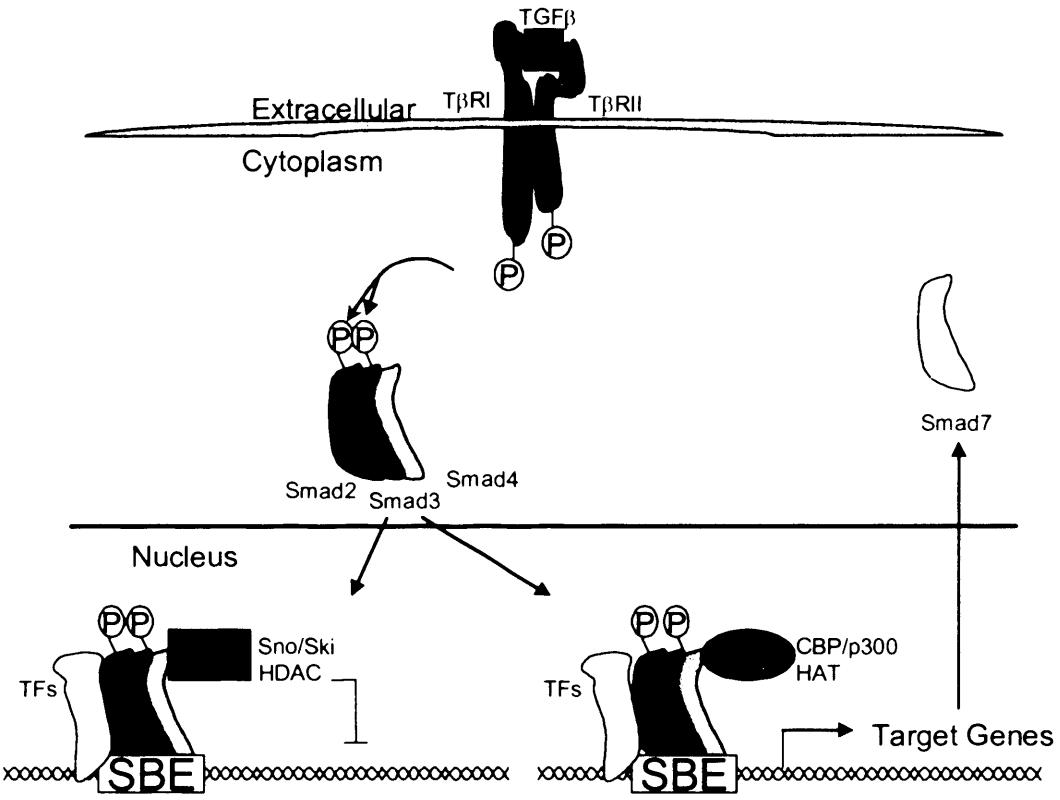


Figure 6.1. Schematic of TGFβ signalling. TGFβ binds to and activates the tetrameric TGFβ receptor complex consisting if TβRI and TβRII. Activated TβRI phosphorylates Smad2 and Smad3 which then complex with Smad4 and translocate to nucleus. The Smad complex associates with other transcription factors and binds to target promoter. It regulates expression via HAT or HDAC. Smad7 is a target of TGFβ and downregulates its signalling by competing with Smad2 and Smad3 for TβRI binding.

Adapted from Li et al., (2006) Ann Rev Immunol 42:99-146

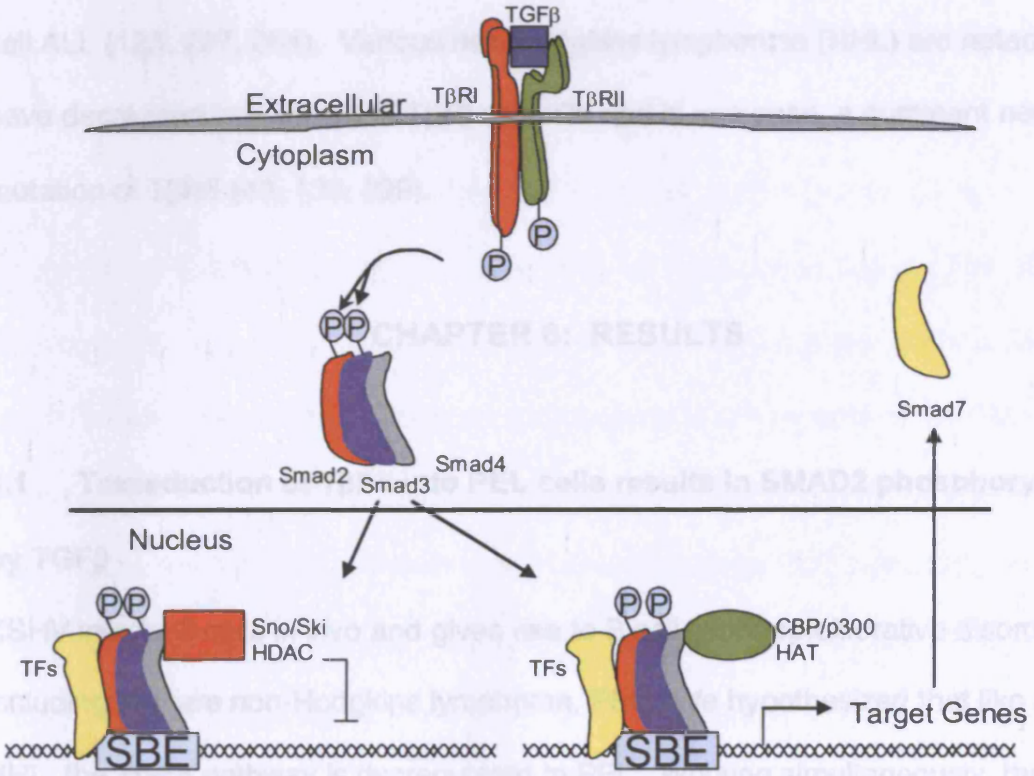


Figure 6.1. Schematic of TGFβ signalling. TGFβ binds to and activates the tetrameric TGFβ receptor complex consisting if TβRI and TβRII. Activated TβRI phosphorylates Smad2 and Smad3 which then complex with Smad4 and translocate to nucleus. The Smad complex associates with other transcription factors and binds to target promoter. It regulates expression via HAT or HDAC. Smad7 is a target of TGFβ and downregulates its signalling by competing with Smad2 and Smad3 for TβRI binding.

Adapted from Li et al., (2006) Ann Rev Immunol 42:99-146

deacetylase through CtBP that interacts with Evi-1(145, 146). Acute myeloid leukemia is associated with dominant-negative mutations in SMAD4 and TβRI polymorphisms, while decreased SMAD3 production has been noted in childhood T-cell ALL (123, 207, 284). Various non-Hodgkins lymphomas (NHL) are noted to have decreased expression of TβRI or TβRII and in one case, a dominant negative mutation of TβRII (40, 138, 239).

CHAPTER 6: RESULTS

6.1 Transduction of TβRII into PEL cells results in SMAD2 phosphorylation by TGFβ

KSHV infects B cells in vivo and gives rise to B cell lymphoproliferative disorders, including the rare non-Hodgkins lymphoma, PEL. We hypothesized that like other NHL, the TGFβ pathway is dysregulated in PEL. Working simultaneously, the Cesarman lab at Cornell and I both found by RT-PCR that PEL cells contain no TβRII message (data not shown). When we realized we were working along the same lines, Dr. Cesarman and I decided to collaborate on this project. In her lab they have found that PEL cells do express the SMADs and that transient transfection of TβRII reconstitutes a transcriptional response to TGFβ. Since B cells are difficult to transfect, phenotypic studies of PEL cells stably expressing TβRII required another approach. I therefore engineered a lentivirus expressing TβRII to transduce the PEL cell line BC3. Figure 6.2A shows that 48 hours after transduction, transduced BC3 cells express TβRII mRNA. Furthermore, this

resulted in SMAD2 phosphorylation in response to TGFβ (5 ng/ml) as demonstrated by the Western blot in 6.2B. Of note, there was a slight but reproducible increase in SMAD2 phosphorylation even in the absence of TGFβ. This is consistent with the Cesarman lab's data that PEL cells secrete TGFβ into the surrounding medium (personal communication).

Using TGFβ-responsive luciferase reporters, I next tested whether reconstitution of TβRII conveyed a transcriptional response to TGFβ. The p3TP-lux construct (gift of J. Massague) is a commonly used TGFβ reporter and the SBE4-luc (gift of Bert Vogelstein) is a similar construct containing 4 repeats of the SMAD-binding element. As seen in Figure 6.3A, increasing doses of TGFβ increase transcription from p3TP-lux construct in TβRII-transduced BC3 cells (top panel) whereas control cells do not respond to even the highest TGFβ dose used (bottom panel). Results were not as pronounced for SBE4-luc (Fig. 6.3B), but they also support the idea that it is the lack of TβRII that renders PEL cells unresponsive to TGFβ.

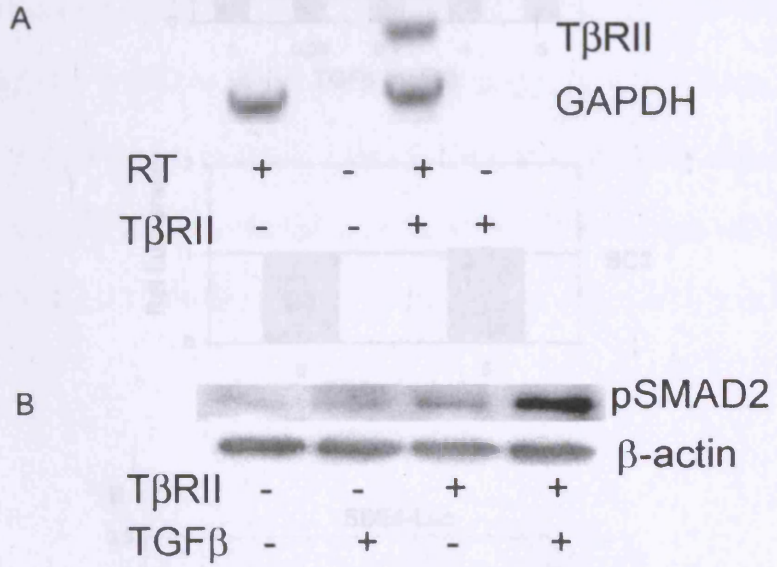


Figure 6.2. Lentiviral transdution of BC3 cells reconstitutes phosphorylation of SMAD2 in presence of TGFβ . BC3 cells were transduced with lenti-TβRII at 5 copies per host cell genome. (**A**) 48 hours post-transduction, mRNA was isolated and RT-PCR performed using primers for both GAPDH and TβRII. Samples without prior RT were also subjected to PCR as control for DNA contamination of mRNA samples. (**B**) BC3 cells with and without lenti-TβRII transduction were stimulated overnight with TGFβ (5ng/ml) where indicated. Western blotting for phosphorylated SMAD2 was then performed on protein lysates. Blots were stripped and re-probed for β-actin as a loading control.

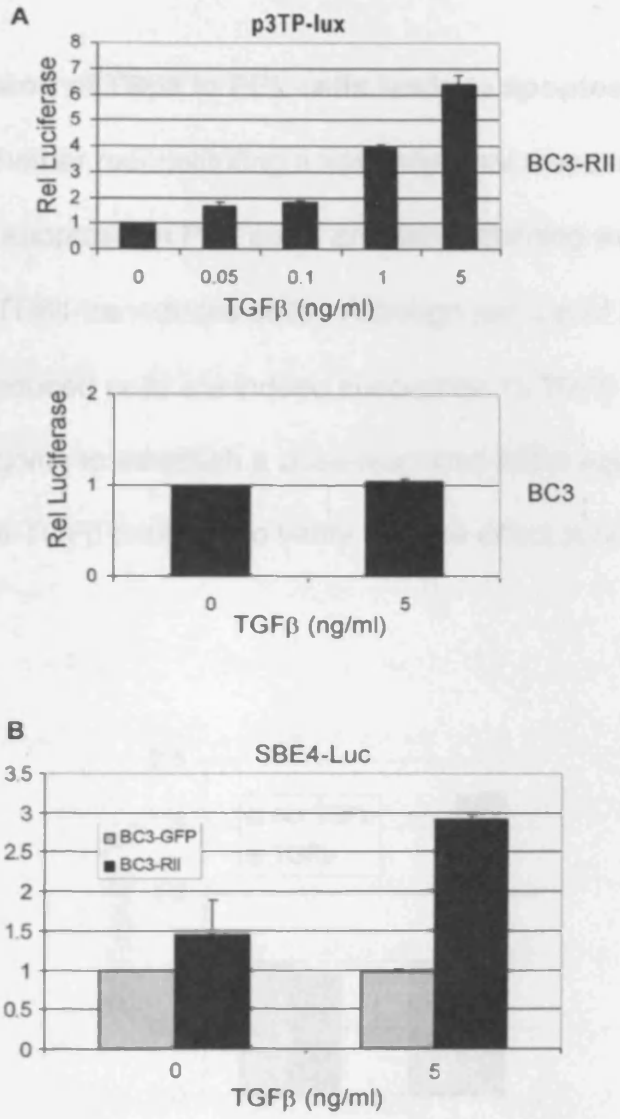


Figure 6.3. Lentiviral transduction of TGFβRII into BC3 cells reconstitutes transcriptional response to TGFβ. (**A**) Top Panel, BC3 cells were transduced with lenti-TGFβRII. 24 hours later they were transfected with the TGFβ luciferase reporter construct p3TP-lux. 24 hours post-transfection cells were exposed to TGFβ at doses shown. Luciferase assays were performed 17 hours later. Lower Panel, BC3 cells transduced with lenti-GFP were transfected with p3TP-lux, exposed to maximum dose of TGFβ, and luciferase assays performed as above. (**B**) BC3 cells transduced with lentivirus expressing either GFP or TGFβRII were transfected with luciferase reporter SBE4-Luc, exposed to TGFβ and subjected to luciferase assays as in (A). Shown are the averages of two experiments each done in triplicate. Results were normalized to control. Bars represent S.D.

6.2 Expression of TRβII in PEL cells leads to apoptosis

To ascertain whether reconstituting a transcriptional respose to TGFβ leads to TGFβ-induced apoptosis in PEL cells, annexin V staining was performed on parent BC3 cells and TRβII-transduced cells. Although just a pilot study, Figure 6.4 shows that TRβII-transduced cells are indeed susceptible to TGFβ-induced apoptosis. Studies are ongoing to establish a dose-response effect and will also incorporate neutralizing anti-TGFβ antibody to verify that the effect seen is due to TGFβ.

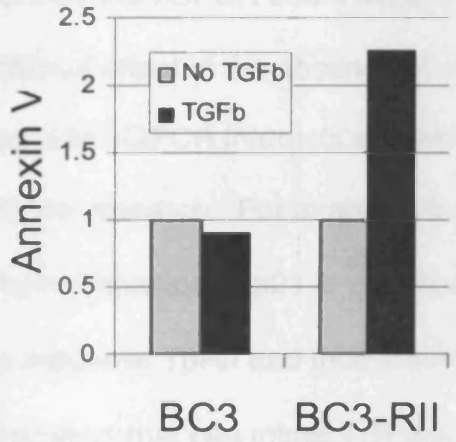


Figure 6.4 Expression of TRβII in PEL cells leads to apoptosis. BC3 cells were transduced with lenti-TRβII or empty lenti vector as control. After 24 hours, cells were exposed to 1 ng/ml TGFβ. 48 hours later annexin V staining was done and quantified by flow cytometry.

6.3 The KSHV vGPCR upregulates transcription via TGFβ-responsive promoters.

The KSHV vGPCR signals via diverse pathways, and as seen in Chapter 3, it upregulates p21 thereby causing cell cycle arrest in PEL cells. Using the p3TP-lux and SBE4-luc reporters shown in Figure 6.3, we tested whether vGPCR could activate a TGFβ-responsive signaling pathway since it too upregulates p21. Figure 6.5 shows that vGPCR increases transcription via both reporters in a dose-dependent manner. To verify specificity of the response, the vGPCR agonist Groα and the inverse agonist, IP-10 were included in the experiment. As would be expected, GROα enhanced the vGPCR effect while IP-10 inhibited it. Neither chemokine had a significant effect in the absence of vGPCR expression. This is a very recent finding, and how vGPCR interacts with the SMAD transcription factors will be the subject of future research. For example, it remains to be established whether vGPCR-mediated induction of p21 is via the same cascade as TGFβ. Why KSHV would evolve to suppress TβRII and thus the normal TGFβ pathway, while encoding its own viral product that can mimic TGFβ will be discussed in Chapter 7.

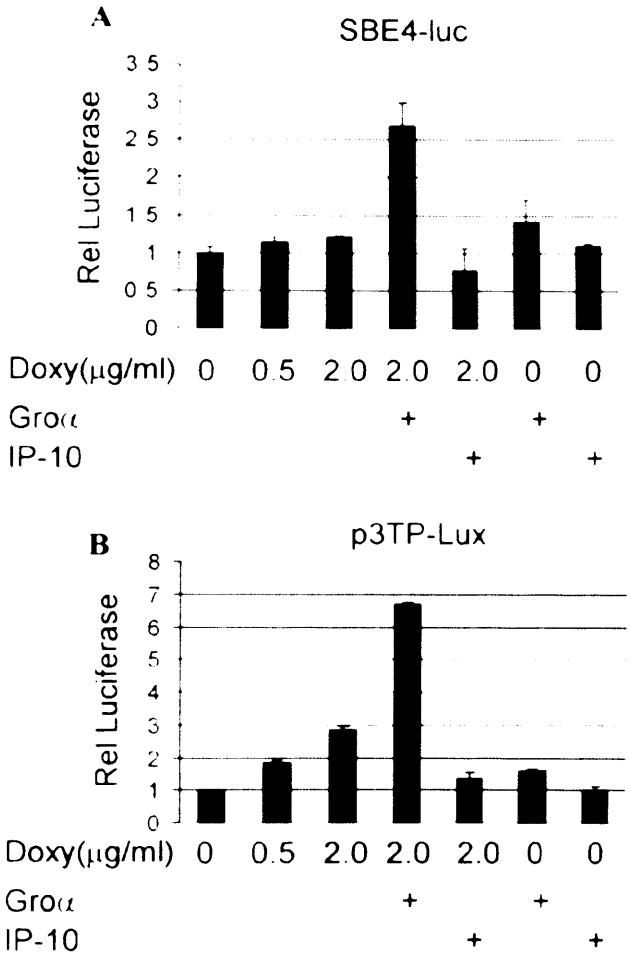


Figure 6.5. KSHV vGPCR activates transcription via TGFβ-responsive promoters in PEL cells. BC3 cells were transfected with either SBE4-luc (**A**), or p3TP-lux (**B**) and then exposed to doses of doxycycline as shown to induce expression of KSHV vGPCR. 24 hours post-transfection, the vGPCR agonist Groα or the inverse agonist IP-10 were added (100nM). 48 hours post-transfection, lysates were made and luciferase assays performed. Shown are averages of two independent experiments. Bars represent S.D.

CHAPTER 7: DISCUSSION

7.1 vGPCR-mediated effects on cell cycle and viral replication

KSHV vGPCR signaling and function have been assayed in several cell types, but few studies have been done in hematopoietic cells. KSHV-infected hematopoietic cells are a vital source of new virion production in all KSHV-mediated disease. Furthermore, ongoing virion production is essential to maintain KSHV-derived tumors. For that reason we studied vGPCR signaling, transcription factor activation and phenotypic effect in PEL cells (KSHV-infected lymphoma cells of B cell lineage). Our initial studies showed that overexpression of vGPCR results in decreased proliferation (37). This finding was surprising since vGPCR had already been classified as a viral oncogene that transforms fibroblasts and endothelial cells. We also noted that when overexpressed in uninduced, latently infected cells, vGPCR modestly increases the transcription of ORF 50 and ORF 57. Since cell cycle manipulation and lytic gene product expression are intimately connected in herpesvirus replication, we sought to better understand the mechanism of vGPCR-induced cell cycle arrest and its effect on lytic gene transcription in KSHV.

Using standard techniques we show that vGPCR signaling results in growth arrest, likely at multiple points throughout the cell cycle. It has been shown that ERK-1/2 and p38 can act in concert to cause a p53-independent, p21-mediated G1 arrest (270). Furthermore, we have previously shown that in PEL cells, vGPCR induces both ERK 1/2 and p38 activity (37). We therefore hypothesized that p21 was the CDI primarily responsible for vGPCR-induced arrest. Our data here

support such a signaling pathway in PEL cells. Consistent with activation of p21, we show that the S-phase kinase Cdk2 is markedly inhibited by vGPCR. Although cell cycle arrest generally coincides with herpesviral latent-lytic switch, Cdk2 activity remains essential for replication (83). Using lytic gene transcription as a surrogate for successful replication, our data strongly support a vGPCR-driven inhibition of viral replication via downregulation of Cdk2. It remains possible however, that there are additional vGPCR-mediated events that also contribute to cell cycle arrest or lytic phase inhibition. For example, it has been shown many times that vGPCR induces NFκB activity; and NFκB has in turn been shown to inhibit the KSHV lytic activation (32).

Several studies support the idea that vGPCR may be expressed outside the context of the KSHV lytic cycle. HIV Tat increases the expression of vGPCR and recent work from the Ganem lab showed that vGPCR may be regulated by RBP-Jκ, a transcription factor and target of the Notch pathway (158, 292). Furthermore, abortive lytic cycle progression in which a subset of lytic genes products is expressed has been shown in other herpesviruses. If such deregulated expression of vGPCR does occur, our data have implications for a longstanding conundrum in the vGPCR literature: as a lytic gene, it is difficult to reconcile either a directly transforming role, or indeed a paracrine role for vGPCR despite the many KS-related cytokines elaborated as a result of its signaling. Presumably a lytically activated KSHV-infected cell that is expressing vGPCR is destined to die and therefore not able to sustain a prolonged paracrine effect on surrounding cells. If, however, a sustained upregulation of vGPCR were to occur prior to a latent-lytic

switch, vGPCR may delay or even prevent full lytic transcription and cell death. Consequently, the proliferative and angiogenic potential of vGPCR would have time to be biologically significant in the tumor microenvironment. Furthermore, vGPCR-driven recruitment of new infectible cells would ensure viral propagation, producing as a byproduct the abnormal cellular proliferation characteristic of KSHV-mediated tumors (Figure 7.1). So although it may be that a few short-lived lytically activated cells can have an effect on the tumor microenvironment, the potential of a vGPCR-induced prolongation of an aberrant lytic phase exists and requires further investigation. The potential effects of a dysregulated (i.e. non-lytically expressed) vGPCR has been described in a recent review by Sodhi et al (251).

Aside from possible dysregulated expression outside the lytic cycle, the 'normal' lytic expression of vGPCR may also play a significant role in KSHV replication. KSHV has evolved mechanisms to carefully regulate vGPCR. Its expression is restricted in that it is transcribed within the 3' end of a bicistronic message; furthermore, KSHV encodes vCLL2, an inverse agonist of vGPCR (267). Precise control of vGPCR signaling suggests that the timing and level of vGPCR signaling is crucial to KSHV propagation and it is likely that vGPCR plays more than one role in course of KSHV-mediated disease. This may explain why we have previously found that in the setting of latent infection, vGPCR overexpression results in modest increased ORF 50 activity (37), but in the setting of lytic induction, vGPCR downwardly modulates the transcription of ORF 50. An interesting finding by Dezube *et. al.* is that during early *de novo* KSHV infection of primary endothelial cells, vGPCR transcription fluctuates in a cyclic pattern with a 48 to 72-hour time

course consistent with viral replication (70). vGPCR-induced changes in viral transcription patterns or in host cell function (including cell cycle) may be required during initial infection to establish successful latency or perhaps to kick start the initial rounds of lytic replication. Whether the cyclic pattern of vGPCR transcription is specific to endothelial cells remains to be studied.

We have established the ability of vGPCR to mediate cell cycle arrest of infected B cells and provide compelling evidence that this occurs via Cdk2 inhibition. Furthermore, we provide initial evidence that this results in abnormal lytic phase induction. Independent inhibition of Cdk2 suggests that as with other herpesviruses, this kinase is required for normal KSHV lytic gene induction. Although this helps explain how a lytic gene product could have important paracrine effects on surrounding cells, there remain questions about the exact role of vGPCR in the establishment of KSHV infection, maintenance of latency, and the latent to lytic switch. Our novel findings presented here are currently being extended to determine how vGPCR expression affects the quantity and infectivity of new virions. Furthermore, vGPCR-mediated effects on other KSHV lytic genes are being investigated and will shed light on the remaining questions about this pirated chemokine receptor and its potential as a target of rationally designed anti-KSHV treatment.

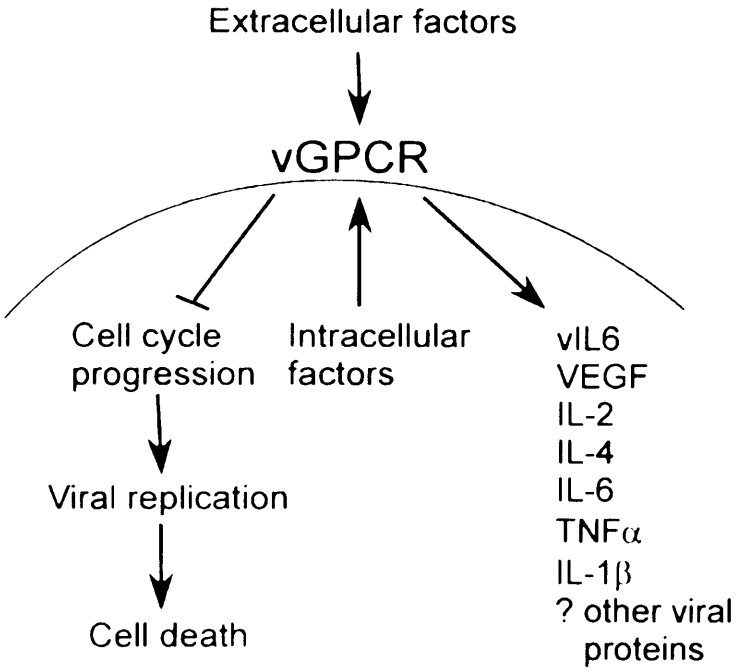


Figure 7.1. Schematic of vGPCR function. Extracellular and intracellular factors both upregulate vGPCR signalling which results in cell cycle arrest with inhibition of viral replication and cell death. This allows more prolonged effect of vGPCR on surrounding cells than would otherwise be possible if the lytic replication phase were to occur unhindered.

7.2 Targeting the PTP pathway

Given that vGPCR is a homologue of human GPCRs, it could be argued that rational therapeutic design to inhibit vGPCR would be unlikely to result in a suitably specific ligand that does not interfere with normal GPCR function. This is a very reasonable argument and so it remains important to discover as much as we can about what role vGPCR plays in different KSHV-infected cell types and which of its many downstream signaling pathways are responsible for these roles. Such knowledge would allow us to direct therapeutic design at specific downstream vGPCR-mediated events. That approach combined with targeted delivery systems would go a long way to achieving acceptable therapeutic indices for novel agents. For these reasons, we extened our search for vGPCR signaling intermediates beyond what was already published. Chapter 5 of this thesis shows some of our initial findings regarding vGPCR and the cytoplasmic PTPs.

The cytoplasmic PTPs, SHP-1 and SHP-2, are involved in signaling via many growth factors and are essential to proper cell proliferation and differentiation. As a testament to their importance, human pathogens from bacteria to protozoa, to viruses have evolved to dysregulate PTP activity: *Helicobacter pylori* activates SHP-2 which in tum dephosphorylates focal adhesion kinase and produces the 'hummingbird' cell shape in gastric epithelial cells (271). Leishmania species interfere with macrophage function and the host immune response by activating SHP-1 (190, 191). HTLV-1 transformed cells have lost SHP-1 activity as have various lymphomas and leukemias (200) (174). Furthermore, overexpression of SHP-2 has been linked to leukemogenesis in humans (290).

Given the strong association of dysregulated PTP function with lymphoma and the known interaction of several GPCRs with the PTPs, we hypothesized that the KSHV vGPCR interacts with this vital signaling pathway. We found that although the consensus ITIM sequence in vGPCR does not result in activation of SHP-2 phosphatase activity, SHP-2 is phosphorylated by vGPCR at its carboxy terminus in a Gαq/Src-dependent manner in HEK 293 cells. A lack of vGPCR-induced SHP-2 phosphatase activity does not mean, however, that SHP-2 is not important to vGPCR signaling. As discussed above, SHP-1 and SHP-2 are thought to have important scaffolding functions aside from their catalytic activity. There are various receptors which associate with SHP-1 and SHP-2 but do not activate their phosphatase activity. Studies are ongoing to determine whether the increased carboxy phosphorylation of SHP-2 results in increased association with Gab1, Gab2, and Grb2, all signaling intermediates capable of binding to SHP-2.

Our experiments so far with SHP-2 have been done in HEK 293 cells. Future work will consist of lentiviral transduction of vGPCR into primary cell types more relevant to KSHV –mediated disease. For example, primary lymphatic endothelial cells (LEC) can be cultured and manipulated in vitro and transduction efficiencies of greater than 50% are achievable with HIV-based lentiviral vectors. Furthermore, both KSHV-infected B cell lines and primary B cells can be readily transduced so that we will be able to determine if vGPCR has the same effects on SHP-2 as we have seen in HEK 293 cells.

Since SHP-1 is expressed predominantly in B cells, we will determine whether KSHV-infected B cells express SHP-1 and if so how vGPCR affects its

binding and/or scaffolding functions. We have very preliminary data that show vGPCR downregulates SHP-1 phosphatase activity (data not shown). As mentioned above, inhibition of SHP-1 expression and/or function is a common event in human lymphomas. It is still unclear whether down-regulation of SHP-1 is itself required for lymphomagenesis or whether low levels of SHP-1 merely reflect the physiologic regulation associated with the immaturity of the neoplastic cells. Clearly, such a conserved mechanism of cell growth dyregulation in haematopoietic malignancies deserves further scrutiny in KSHV-associated lymphoproliferative diseases. It is possible that KSHV requires vGPCR to inhibit SHP-1 at certain times in the process of viral replication or perhaps to give a growth advantage to KSHV-infected B cells that are mature enough to be expressing SHP-1. Luckily, both gain and loss-of-function mutants are available for SHP-1 and SHP-2 and will facilitate exploring how vGPCR manipulates the PTPs and which downstream vGPCR events rely on that manipulation. Only then can we determine if interfering with vGPCR-induced changes in PTP function is a feasible anti-KSHV strategy.

7.3 Dysregulation of TGFβ pathway in KSHV-mediated disease

The pathogenesis and treatment of haematopoietic malignancies differs somewhat from that of solid tumours. The former are more associated with aging whereas the latter affect a broad age range. Of course similarities exist and include the ability to resist growth-inhibitory and differentiation factors, growth in the absence of exogenous growth signals, evasion of apoptosis, and the evasion of immune surveillance. The TGFβ pathway is an example of a homeostatic mechanism that is frequently disrupted in haematopoietic malignancies. We therefore sought to establish whether such is the case in PEL, one of the KSHV-mediated lymphoproliferative diseases.

Together with the Cesarman lab at Cornell, we found that none of the PEL lines tested express TβRII and therefore cannot signal in response to TGFβ. In this thesis I used lentiviral transduction to reconstitute TβRII expression and show that in transduced cells, TGFβ leads to SMAD2 phosphorylation whereas the untransduced cells show none. Using luciferase reporter constructs I also show that transduction of TβRII leads to transcriptional activation of TGFβ and SMAD-responsive genes in response to TGFβ. Together these results strongly suggest that other than the TβRII, the components of the TGFβ pathway are intact in PEL cells. Furthermore, the Cesarman lab has confirmed the expression of the SMADs by rt-PCR (personal communication). To test whether transduction of TRβII leads to a phenotypic response to TGFβ, I have begun apoptosis assays in which parent BC3 cells and TRβII-transduced BC3 cells are compared in their apoptotic response

to TGFβ as evidenced by annexin V staining. Although these experiments are still underway, the pilot study suggests that this is the case. The experiment is being optimized and eventually will be performed with escalating doses of TGFβ as well as neutralizing anti-TGFβ antibody to ensure that any differences between transduced and untransduced cells are specifically due to TGFβ.

As noted in Chapter 6, it appears that PEL cells secrete TGFβ into their surrounding medium. This fact combined with our findings that PEL cells are unresponsive to TGFβ suggests that KSHV has evolved a survival strategy that can potentially confer a growth advantage to infected haematopoietic cells while inhibiting the host immune response. Since TGFβ is homeostatic for most cells of haematopoietic lineage, KSHV-infected B cells need to be resistent to its effects. Furthermore, since some degree of KSHV replication is always ongoing, the virus has needed to evolve varied and potent immune evasion strategies. It is highly plausible that by secreting TGFβ, KSHV-infected cells discourage the proliferation and differentiation of local host immune cells.

Future work will include using a well established mouse model of PEL in which intraperitoneal injection of PEL cells results in gross ascites (133). Using this model, lentiviral transduction of TβRII can be assessed for its effect on PEL development as determined by serial girth measurements. Such experiments could further support the hypothesis that downregulation of TβRII is a potent survival mechanism in KSHV-induced lymphoproliferative disease.

As presented in Figure 6.5 we have found that KSHV vGPCR may share some downstream effectors with the TGFβ pathway. vGPCR transfection into PEL cells activates both TGFβ- and SMAD-responsive promoters. Whether vGPCR uses the same mechanism as TGFβ to upregulate p21 (see Chapter 3) remains to be seen. Furthermore, we and others have shown that vGPCR upregulates the secretion of both human IL-6 and viral IL-6 from various haematopoietic cell lines. TGFβ and IL-6 together are known to have effects on T cell differentiation. For example, the TGFβ elaborated by Treg cells along with IL-6 subvert Th1 and Th2 development in favor of the proinflammatory IL-17 secreting T17 cells (275, 276). Whether vGCPR is involved in subverting the immune response has not been properly investigated but our new data have prompted us to characterize the potential autocrine and paracrine effects of vGPCR on T cell differentiation. Autocrine effects can be studied using lentiviral transduction of vGPCR; paracrine effects using the conditioned medium of PEL cells +/- overexpression of vGPCR will also be studied. Chemokine receptors help co-ordinate both the innate and adaptive immune responses, so further study of vGPCR in this context is likely to very informative.

7.4 Conclusions

During its evolution, KSHV has pirated several genes from its primate hosts. These have generally undergone mutation, presumably conferring a proliferative advantage to the virus. In the case of vGPCR, the advantages appear to be many. We know that vGPCR expression confers both an autocrine and paracrine growth advantage to endothelial cells. Furthermore, expression in haematopoietic cells results in the elaboration of many cytokines that have long been associated with KSHV-mediated diseases. In this thesis we have presented evidence that vGPCR also has a negative effect on host cell cycle which in turn inhibits viral production from B cell reservoirs. We hypothesize that this inhibition of virion production may lend additional potency to the paracrine effects of vGPCR by creating a population of infected cells that secrete cytokines required for angiogenesis and recruitment of infectable cells necessary to viral reproduction. Since vGPCR is a chemokine receptor it is also likely that it has an effect on the host immune response. Our preliminary data showing that vGPCR can mimic TGFβ signaling in reporter assays supports this notion and requires further study.

REFERENCES

1. Adachi, M., E. H. Fischer, J. Ihle, K. Imai, F. Jirik, B. Neel, T. Pawson, S. Shen, M. Thomas, A. Ullrich, and Z. Zhao. 1996. Mammalian SH2-containing protein tyrosine phosphatases. Cell 85:15.
2. Ahuja, S. K., and P. M. Murphy. 1993. Molecular piracy of mammalian interleukin-8 receptor type B by herpesvirus saimiri. J Biol Chem 268:20691-4.
3. Akashi, M., Y. Osawa, H. P. Koeffler, and M. Hachiya. 1999. p21WAF1 expression by an activator of protein kinase C is regulated mainly at the post-transcriptional level in cells lacking p53: important role of RNA stabilization. Biochem J 337 (Pt 3):607-16.
4. Alonso, A., J. Sasin, N. Bottini, I. Friedberg, A. Osterman, A. Godzik, T. Hunter, J. Dixon, and T. Mustelin. 2004. Protein tyrosine phosphatases in the human genome. Cell 117:699-711.
5. Aoki, Y., E. S. Jaffe, Y. Chang, K. Jones, J. Teruya-Feldstein, P. S. Moore, and G. Tosato. 1999. Angiogenesis and hematopoiesis induced by Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. Blood 93:4034-43.
6. Aoki, Y., and G. Tosato. 1999. Role of vascular endothelial growth factor/vascular permeability factor in the pathogenesis of Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphomas. Blood 94:4247-54.

7. Aoki, Y., and G. Tosato. 2001. Vascular endothelial growth factor/vascular permeability factor in the pathogenesis of primary effusion lymphomas. Leuk Lymphoma 41:229-37.

8. Arvanitakis, L., E. Geras-Raaka, and M. C. Gershengorn. 1998. Constitutively Signaling G-Protein-Coupled Receptors and Human Disease. TEM 9:27-31.

9. Arvanitakis, L., E. Geras-Raaka, A. Varma, M. C. Gershengorn, and E. Cesarman. 1997. Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. Nature 385:347-50.

10. Arvanitakis, L., E. A. Mesri, R. G. Nador, J. W. Said, A. S. Asch, D. M. Knowles, and E. Cesarman. 1996. Establishment and characterization of a primary effusion (body cavity-based) lymphoma cell line (BC-3) harboring kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in the absence of Epstein-Barr virus. Blood 88:2648-54.

11. Ascoli, V., C. M. Mastroianni, V. Galati, M. C. Sirianni, A. Fruscalzo, A. Pistilli, and F. Lo Coco. 1998. Primary effusion lymphoma containing human herpesvirus 8 DNA in two AIDS patients with Kaposi's sarcoma. Haematologica 83:8-12.

12. Attisano, L., and J. L. Wrana. 2002. Signal transduction by the TGF-beta superfamily. Science 296:1646-7.

13. Bais, C., B. Santomasso, O. Coso, L. Arvanitakis, E. G. Raaka, J. S. Gutkind, A. S. Asch, E. Cesarman, M. C. Gershengorn, and E. A. Mesri. 1998. G-

protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. Nature 391:86-9.

14. Bais, C., A. Van Geelen, P. Eroles, A. Mutlu, C. Chiozzini, S. Dias, R. L. Silverstein, S. Rafii, and E. A. Mesri. 2003. Kaposi's sarcoma associated herpesvirus G protein-coupled receptor immortalizes human endothelial cells by activation of the VEGF receptor-2/ KDR. Cancer Cell 3:131-43.

15. Barak, Y., T. Juven, R. Haffner, and M. Oren. 1993. mdm2 expression is induced by wild type p53 activity. Embo J 12:461-8.

16. Barillari, G., R. Gendelman, R. C. Gallo, and B. Ensoli. 1993. The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. Proc Natl Acad Sci U S A 90:7941-5.

17. Barozzi, P., M. Luppi, L. Msini, R. Marasca, M. Savarino, M. Morselli, M. Ferrari, M. Bevini, G. Bonacorsi, and G. Torelli. 1996. J. Clin Pathol Mol Pathol 49:M232-M235.

18. Beisser, P. S., D. Verzijl, Y. K. Gruijthuijsen, E. Beuken, M. J. Smit, R. Leurs, C. A. Bruggeman, and C. Vink. 2005. The Epstein-Barr virus BILF1 gene encodes a G protein-coupled receptor that inhibits phosphorylation of RNA-dependent protein kinase. J Virol 79:441-9.

19. Beisser, P. S., C. Vink, J. G. Van Dam, G. Grauls, S. J. Vanherle, and C. A. Bruggeman. 1998. The R33 G protein-coupled receptor gene of rat

cytomegalovirus plays an essential role in the pathogenesis of viral infection. J Virol 72:2352-63.

20. Benelli, R., A. Albini, C. Parravicini, S. Carlone, L. Repetto, G. Tambussi, and A. Lazzarin. 1996. Isolation of spindle-shaped cell populations from primary cultures of Kaposi's sarcoma of different stage. Cancer Lett 100:125-32.

21. Biggar, R. J., R. E. Curtis, T. R. Cote, C. S. Rabkin, and M. Melbye. 1994. Risk of other cancers following Kaposi's sarcoma: relation to acquired immunodeficiency syndrome. Am J Epidemiol 139:362-8.

22. Blasig, C., C. Zietz, B. Haar, F. Neipel, S. Esser, N. H. Brockmeyer, E. Tschachler, S. Colombini, B. Ensoli, and M. Sturzl. 1997. Monocytes in Kaposi's sarcoma lesions are productively infected by human herpesvirus 8. J Virol 71:7963-8.

23. Bockaert, J., and J. P. Pin. 1999. Molecular tinkering of G protein-coupled receptors: an evolutionary success. Embo J 18:1723-9.

24. Bodaghi, B., T. R. Jones, D. Zipeto, C. Vita, L. Sun, L. Laurent, F. Arenzana-Seisdedos, J. L. Virelizier, and S. Michelson. 1998. Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus-infected cells. J Exp Med 188:855-66.

25. Bogdan, C., J. Paik, Y. Vodovotz, and C. Nathan. 1992. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. J Biol Chem 267:23301-8.

26. Bornkamm, G. W., H. Delius, B. Fleckenstein, F. J. Werner, and C. Mulder. 1976. Structure of Herpesvirus saimiri genomes: arrangement of heavy and light sequences in the M genome. J Virol 19:154-61.

27. Boshoff, C., Y. Endo, P. D. Collins, Y. Takeuchi, J. D. Reeves, V. L. Schweickart, M. A. Siani, T. Sasaki, T. J. Williams. P. W. Gray, P. S. Moore, Y. Chang, and R. A. Weiss. 1997. Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. Science 278:290-4.

28. Boshoff, C., T. F. Schulz, M. M. Kennedy, A. K. Graham, C. Fisher, A. Thomas, J. O. McGee, R. A. Weiss, and O. L. JJ. 1995. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat Med 1:1274-8.

29. Bresnahan, W. A., I. Boldogh, P. Chi, E. A. Thompson, and T. Albrecht. 1997. Inhibition of cellular Cdk2 activity blocks human cytomegalovirus replication. Virology 231:239-47.

30. Bresnahan, W. A., E. A. Thompson, and T. Albrecht. 1997. Human cytomegalovirus infection results in altered Cdk2 subcellular localization. J Gen Virol 78 (Pt 8):1993-7.

31. Brink, C. B., B. H. Harvey, J. Bodenstein, D. P. Venter, and D. W. Oliver. 2004. Recent advances in drug action and therapeutics: relevance of novel concepts in G-protein-coupled receptor and signal transduction pharmacology. Br J Clin Pharmacol 57:373-87.

32. Brown, H. J., M. J. Song, H. Deng, T. T. Wu, G. Cheng, and R. Sun. 2003. NF-kappaB inhibits gammaherpesvirus lytic replication. J Virol 77:8532-40.

33. Buchkovich, K., L. A. Duffy, and E. Harlow. 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58:1097-105.

34. Burns, J. C., T. Friedmann, W. Driever, M. Burrascano, and J. K. Yee. 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc Natl Acad Sci U S A 90:8033-7.

35. Burshtyn, D. N., A. M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, T. Yi, J. P. Kinet, and E. O. Long. 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. Immunity 4:77-85.

36. Burysek, L., W. S. Yeow, B. Lubyova, M. Kellum, S. L. Schafer, Y. Q. Huang, and P. M. Pitha. 1999. Functional analysis of human herpesvirus 8-encoded viral interferon regulatory factor 1 and its association with cellular interferon regulatory factors and p300. J Virol 73:7334-42.

37. Cannon, M., N. J. Philpott, and E. Cesarman. 2003. The Kaposi's Sarcoma-Associated Herpesvirus G Protein-Coupled Receptor Has Broad Signaling Effects in Primary Effusion Lymphoma Cells. J Virol 77:57-67.

38. Cannon, M. L., and E. Cesarman. 2004. The KSHV G protein-coupled receptor signals via multiple pathways to induce transcription factor activation in primary effusion lymphoma cells. Oncogene 23:514-23.

39. Cantaluppi, V., L. Biancone, M. Boccellino, S. Doublier, R. Benelli, S. Carlone, A. Albini, and G. Camussi. 2001. HIV type 1 Tat protein is a survival factor for Kaposi's sarcoma and endothelial cells. AIDS Res Hum Retroviruses 17:965-76.

40. Capocasale, R. J., R. J. Lamb, E. C. Vonderheid, F. E. Fox, A. H. Rook, P. C. Nowell, and J. S. Moore. 1995. Reduced surface expression of transforming growth factor beta receptor type II in mitogen-activated T cells from Sezary patients. Proc Natl Acad Sci U S A 92:5501-5.

41. Casarosa, P., R. A. Bakker, D. Verzijl, M. Navis, H. Timmerman, R. Leurs, and M. J. Smit. 2001. Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. J Biol Chem 276:1133-7.

42. Casarosa, P., Y. K. Gruijthuijsen, D. Michel, P. S. Beisser, J. Holl, C. P. Fitzsimons, D. Verzijl, C. A. Bruggeman, T. Mertens, R. Leurs, C. Vink, and M. J. Smit. 2003. Constitutive signaling of the human cytomegalovirus-encoded receptor UL33 differs from that of its rat cytomegalovirus homolog R33 by promiscuous activation of G proteins of the Gq, Gi, and Gs classes. J Biol Chem 278:50010-23.

43. Cesarman, E., A. Chadburn, Y. F. Liu, A. Migliazza, R. Dalla-Favera, and D. M. Knowles. 1998. BCL-6 gene mutations in posttransplantation lymphoproliferative disorders predict response to therapy and clinical outcome. Blood 92:2294-302.

44. Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med 332:1186-91.

45. Cesarman, E., and D. M. Knowles. 1997. Kaposi's sarcoma-associated herpesvirus: a lymphotropic human herpesvirus associated with Kaposi's

sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Semin Diagn Pathol 14:54-66.

46. Cesarman, E., and D. M. Knowles. 1999. The role of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in lymphoproliferative diseases. Semin Cancer Biol 9:165-74.

47. Cesarman, E., P. S. Moore, P. H. Rao, G. Inghirami, D. M. Knowles, and Y. Chang. 1995. In vitro establishment and characterization of two acquired immunodeficiency syndrome-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. Blood 86:2708-14.

48. Cesarman, E., R. G. Nador, F. Bai, R. A. Bohenzky, J. J. Russo, P. S. Moore, Y. Chang, and D. M. Knowles. 1996. Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. J Virol 70:8218-23.

49. Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore. 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 266:1865-9.

50. Chang, Y., P. S. Moore, S. J. Talbot, C. H. Boshoff, T. Zarkowska, K. Godden, H. Paterson, R. A. Weiss, and S. Mittnacht. 1996. Cyclin encoded by KS herpesvirus [letter]. Nature 382:410.

51. Chang, Y., J. Ziegler, H. Wabinga, E. Katangole-Mbidde, C. Boshoff, T. Schulz, D. Whitby, D. Maddalena, H. W. Jaffe, R. A. Weiss, and P. S. Moore.

1996. Kaposi's sarcoma-associated herpesvirus and Kaposi's sarcoma in Africa. Uganda Kaposi's Sarcoma Study Group. Arch Intern Med 156:202-4.

52. Chattopadhyay, N., A. Mithal, and E. M. Brown. 1996. The calcium-sensing receptor: a window into the physiology and pathophysiology of mineral ion metabolism. Endocr Rev 17:289-307.

53. Chee, M., and B. Barrell. 1990. Herpesviruses: a study of parts. Trends Genet 6:86-91.

54. Chen, C. R., Y. Kang, P. M. Siegel, and J. Massague. 2002. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. Cell 110:19-32.

55. Chen, H. E., S. Chang, T. Trub, and B. G. Neel. 1996. Regulation of colony-stimulating factor 1 receptor signaling by the SH2 domain-containing tyrosine phosphatase SHPTP1. Mol Cell Biol 16:3685-97.

56. Chen, P. L., P. Scully, J. Y. Shew, J. Y. Wang, and W. H. Lee. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58:1193-8.

57. Cheng, E. H., J. Nicholas, D. S. Bellows, G. S. Hayward, H. G. Guo, M. S. Reitz, and J. M. Hardwick. 1997. A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. Proc Natl Acad Sci U S A 94:690-4.

58. Chiou, C. J., L. J. Poole, P. S. Kim, D. M. Ciufo, J. S. Cannon, C. M. ap Rhys, D. J. Alcendor, J. C. Zong, R. F. Ambinder, and G. S. Hayward. 2002. Patterns of Gene Expression and a Transactivation Function Exhibited by the

vGCR (ORF74) Chemokine Receptor Protein of Kaposi's Sarcoma-Associated Herpesvirus. J Virol 76:3421-39.

59. Civati, G., G. Busnach, B. Brando, M. L. Broggi, C. Brunati, G. P. Casadei, and L. Minetti. 1988. Occurrence of Kaposi's sarcoma in renal transplant recipients treated with low doses of cyclosporine. Transplant Proc 20:924-8.

60. Clark, D. A., V. C. Emery, and P. D. Griffiths. 2003. Cytomegalovirus, human herpesvirus-6, and human herpesvirus-7 in hematological patients. Semin Hematol 40:154-62.

61. Coughlin, S. R. 1994. Expanding horizons for receptors coupled to G proteins: diversity and disease. Curr Opin Cell Biol 6:191-7.

62. Couty, J. P., E. Geras-Raaka, B. B. Weksler, and M. C. Gershengorn. 2001. Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor signals through multiple pathways in endothelial cells. J Biol Chem 276:33805-11.

63. D'Ambrosio, D., D. C. Fong, and J. C. Cambier. 1996. The SHIP phosphatase becomes associated with Fc gammaRIIB1 and is tyrosine phosphorylated during 'negative' signaling. Immunol Lett 54:77-82.

64. D'Ambrosio, D., K. L. Hippen, S. A. Minskoff, I. Mellman, G. Pani, K. A. Siminovitch, and J. C. Cambier. 1995. Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by Fc gamma RIIB1. Science 268:293-7.

65. Davison, A. J., and J. E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. J Gen Virol 67 (Pt 9):1759-816.

66. Davison, A. J., and P. Taylor. 1987. Genetic relations between varicella-zoster virus and Epstein-Barr virus. J Gen Virol 68 (Pt 4):1067-79.

67. Demaison, C., K. Parsley, G. Brouns, M. Scherr, K. Battmer, C. Kinnon, M. Grez, and A. J. Thrasher. 2002. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum Gene Ther 13:803-13.

68. Derynck, R., and X. H. Feng. 1997. TGF-beta receptor signaling. Biochim Biophys Acta 1333:F105-50.

69. Derynck, R., and Y. E. Zhang. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425:577-84.

70. Dezube, B. J., M. Zambela, D. R. Sage, J. F. Wang, and J. D. Fingeroth. 2002. Characterization of Kaposi sarcoma-associated herpesvirus/human herpesvirus-8 infection of human vascular endothelial cells: early events. Blood 100:888-96.

71. Duchene, J., J. P. Schanstra, C. Pecher, A. Pizard, C. Susini, J. P. Esteve, J. L. Bascands, and J. P. Girolami. 2002. A novel protein-protein interaction between a G protein-coupled receptor and the phosphatase SHP-2 is involved in bradykinin-induced inhibition of cell proliferation. J Biol Chem 277:40375-83.

72. Dupin, N., C. Fisher, P. Kellam, S. Ariad, M. Tulliez, N. Franck, E. van Marck, D. Salmon, I. Gorin, J. P. Escande, R. A. Weiss, K. Alitalo, and C. Boshoff. 1999. Distribution of human herpesvirus-8 latently infected cells in Kaposi's

sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Proc Natl Acad Sci U S A 96:4546-51.

73. Dupin, N., I. Gorin, J. Deleuze, H. Agut, J. M. Huraux, and J. P. Escande. 1995. Herpes-like DNA sequences, AIDS-related tumors, and Castleman's disease [letter; comment]. N Engl J Med 333:798; discussion 798-9.

74. Ensoli, B., L. Buonaguro, G. Barillari, V. Fiorelli, R. Gendelman, R. A. Morgan, P. Wingfield, and R. C. Gallo. 1993. Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. J Virol 67:277-87.

75. Ensoli, B., R. Gendelman, P. Markham, V. Fiorelli, S. Colombini, M. Raffeld, A. Cafaro, H. K. Chang, J. N. Brady, and R. C. Gallo. 1994. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature 371:674-80.

76. Ensoli, B., P. Markham, V. Kao, G. Barillari, V. Fiorelli, R. Gendelman, M. Raffeld, G. Zon, and R. C. Gallo. 1994. Block of AIDS-Kaposi's sarcoma (KS) cell growth, angiogenesis, and lesion formation in nude mice by antisense oligonucleotide targeting basic fibroblast growth factor. A novel strategy for the therapy of KS. J Clin Invest 94:1736-46.

77. Ensoli, B., P. Monini, and C. Sgadari. 1999. Pathogenesis and Cell Biology of Kaposi's Sarcoma, p. 385-413. *In* A. Dalgleish and R. Weiss (ed.), HIV and the New Viruses. Academic Press, San Diego.

78. Ensoli, B., S. Nakamura, S. Z. Salahuddin, P. Biberfeld, L. Larsson, B. Beaver, F. Wong-Staal, and R. C. Gallo. 1989. AIDS-Kaposi's sarcoma-

derived cells express cytokines with autocrine and paracrine growth effects. Science 243:223-6.

79. Federman, A. D., B. R. Conklin, K. A. Schrader, R. R. Reed, and H. R. Bourne. 1992. Hormonal stimulation of adenylyl cyclase through Gi-protein beta gamma subunits. Nature 356:159-61.

80. Feng, G. S., C. C. Hui, and T. Pawson. 1993. SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. Science 259:1607-11.

81. Feng, Y. H., Y. Sun, and J. G. Douglas. 2002. Gbeta gamma -independent constitutive association of Galpha s with SHP-1 and angiotensin II receptor AT2 is essential in AT2-mediated ITIM-independent activation of SHP-1. Proc Natl Acad Sci U S A 99:12049-54.

82. Flaitz, C. M., Y. T. Jin, M. J. Hicks, C. M. Nichols, Y. W. Wang, and I. J. Su. 1997. Kaposi's sarcoma-associated herpesvirus-like DNA sequences (KSHV/HHV-8) in oral AIDS-Kaposi's sarcoma: a PCR and clinicopathologic study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 83:259-64.

83. Flemington, E. K. 2001. Herpesvirus lytic replication and the cell cycle: arresting new developments. J Virol 75:4475-81.

84. Flore, O., and S. J. Gao. 1997. Effect of DNA synthesis inhibitors on Kaposi's sarcoma-associated herpesvirus cyclin and major capsid protein gene expression. AIDS Res Hum Retroviruses 13:1229-33.

85. Flotte, T. R., and B. J. Carter. 1995. Adeno-associated virus vectors for gene therapy. Gene Ther 2:357-62.

86. Flowers, C. C., S. Flowers, and G. Nabel. 1998. Kaposi's Sarcoma-Associated Herpesvirus Viral Interferon Regulatory Factor Confers Resistance to the Antiproliferative Effect of Interferon-alpha. Mol Med 4:402-412.

87. Fortunel, N. O., A. Hatzfeld, and J. A. Hatzfeld. 2000. Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. Blood 96:2022-36.

88. Franceschi, S., and M. Geddes. 1995. Epidemiology of classic Kaposi's sarcoma, with special reference to mediterranean population. Tumori 81:308-14.

89. Friedman-Kien, A. E. 1981. Disseminated Kaposi's sarcoma syndrome in young homosexual men. J Am Acad Dermatol 5:468-71.

90. Frizzera, G. 1992. Atypical lymphoproliferative disorders, p. 459-496. *In* D. M. Knowles (ed.), Neoplastic Hematopathology. Williams and Wilkins, Baltimore, MD.

91. Gaidano, G., K. Cechova, Y. Chang, P. S. Moore, D. M. Knowles, and R. Dalla-Favera. 1996. Establishment of AIDS-related lymphoma cell lines from lymphomatous effusions. Leukemia 10:1237-40.

92. Gallo, R. C. 1998. Some aspects of the pathogenesis of HIV-1-associated Kaposi's sarcoma. J Natl Cancer Inst Monogr:55-7.

93. Gao, S. J., C. Boshoff, S. Jayachandra, R. A. Weiss, Y. Chang, and P. S. Moore. 1997. KSHV ORF K9 (vIRF) is an oncogene which inhibits the interferon signaling pathway. Oncogene 15:1979-85.

94. Gao, S. J., L. Kingsley, D. R. Hoover, T. J. Spira, C. R. Rinaldo, A. Saah, J. Phair, R. Detels, P. Parry, Y. Chang, and P. S. Moore. 1996. Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. N Engl J Med 335:233-41.

95. Garrigue-Antar, L., T. Munoz-Antonia, S. J. Antonia, J. Gesmonde, V. F. Vellucci, and M. Reiss. 1995. Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. Cancer Res 55:3982-7.

96. Gartel, A. L., and A. L. Tyner. 1999. Transcriptional regulation of the p21((WAF1/CIP1)) gene. Exp Cell Res 246:280-9.

97. Geras-Raaka, E., L. Arvanitakis, C. Bais, E. Cesarman, E. A. Mesri, and M. C. Gershengorn. 1998. Inhibition of constitutive signaling of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor by protein kinases in mammalian cells in culture. J Exp Med 187:801-6.

98. Geras-Raaka, E., A. Varma, I. Clark-Lewis, and M. Gershengorn. 1998. Kaposi's sarcoma-associated herpesvirus (KSHV) chemokine vMIP-II and human SDF-1alpha inhibit signaling by KSHV G protein-coupled receptor. Biochem Biophys Res Commun 253: p725-7.

99. Geras-Raaka, E., A. Varma, H. Ho, I. Clark-Lewis, and M. C. Gershengorn. 1998. Human interferon-gamma-inducible protein 10 (IP-10) inhibits constitutive signaling of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. J Exp Med 188:405-8.

100. Gershengorn, M. C., E. Geras-Raaka, A. Varma, and I. Clark-Lewis. 1998. Chemokines activate Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor in mammalian cells in culture. J Clin Invest 102:1469-72.

101. Gershengorn, M. C., and R. Osman. 2001. Minireview: Insights into G Protein-Coupled Receptor Function Using Molecular Models. [Record Supplied By Publisher]. Endocrinology 142:p2-10.

102. Gill, P. S., Y. C. Tsai, A. P. Rao, C. H. Spruck, 3rd, T. Zheng, W. A. Harrington, Jr., T. Cheung, B. Nathwani, and P. A. Jones. 1998. Evidence for multiclonality in multicentric Kaposi's sarcoma. Proc Natl Acad Sci U S A 95:8257-61.

103. Gompels, U. A., J. Nicholas, G. Lawrence, M. Jones, B. J. Thomson, M. E. Martin, S. Efstathiou, M. Craxton, and H. A. Macaulay. 1995. The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. Virology 209:29-51.

104. Gorelik, L., and R. A. Flavell. 2002. Transforming growth factor-beta in T-cell biology. Nat Rev Immunol 2:46-53.

105. Gossen, M., A. L. Bonin, S. Freundlieb, and H. Bujard. 1994. Inducible gene expression systems for higher eukaryotic cells. Curr Opin Biotechnol 5:p516-20.

106. Gossen, M., S. Freundlieb, G. Bender, G. Muller, W. Hillen, and H. Bujard. 1995. Transcriptional activation by tetracyclines in mammalian cells. Science 268:p1766-9.

107. Goumans, M. J., G. Valdimarsdottir, S. Itoh, A. Rosendahl, P. Sideras, and P. ten Dijke. 2002. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. Embo J 21:1743-53.

108. Grana, X., J. Garriga, and X. Mayol. 1998. Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. Oncogene 17:3365-83.

109. Grandadam, M., N. Dupin, V. Calvez, I. Gorin, L. Blum, S. Kernbaum, D. Sicard, Y. Buisson, H. Agut, J. P. Escande, and J. M. Huraux. 1997. Exacerbations of clinical symptoms in human immunodeficiency virus type 1-infected patients with multicentric Castleman's disease are associated with a high increase in Kaposi's sarcoma herpesvirus DNA load in peripheral blood mononuclear cells. J Infect Dis 175:1198-201.

110. Green, I., E. Espiritu, M. Ladanyi, R. Chaponda, R. Wieczorek, L. Gallo, and H. Feiner. 1995. Primary lymphomatous effusions in AIDS: a morphological, immunophenotypic, and molecular study. Mod Pathol 8:39-45.

111. Guo, H. G., P. Browning, J. Nicholas, G. S. Hayward, E. Tschachler, Y. W. Jiang, M. Sadowska, M. Raffeld, S. Colombini, R. C. Gallo, and M. S. Reitz, Jr. 1997. Characterization of a chemokine receptor-related gene in human herpesvirus 8 and its expression in Kaposi's sarcoma. Virology 228:371-8.

112. Guo, H. G., S. Pati, M. Sadowska, M. Charurat, and M. Reitz. 2004. Tumorigenesis by human herpesvirus 8 vGPCR is accelerated by human immunodeficiency virus type 1 Tat. J Virol 78:9336-42.

113. Guo, H. G., M. Sadowska, W. Reid, E. Tschachler, G. Hayward, and M. Reitz. 2003. Kaposi's sarcoma-like tumors in a human herpesvirus 8 ORF74 transgenic mouse. J Virol 77:2631-9.

114. Guo, Y., S. C. Jacobs, and N. Kyprianou. 1997. Down-regulation of protein and mRNA expression for transforming growth factor-beta (TGF-beta1) type I and type II receptors in human prostate cancer. Int J Cancer 71:573-9.

115. Gupta, P., M. K. Singh, C. Rinaldo, M. Ding, H. Farzadegan, A. Saah, D. Hoover, P. Moore, and L. Kingsley. 1996. Detection of Kaposi's sarcoma herpesvirus DNA in semen of homosexual men with Kaposi's sarcoma [letter]. Aids 10:1596-8.

116. Hall, R. A., and R. J. Lefkowitz. 2002. Regulation of G protein-coupled receptor signaling by scaffold proteins. Circ Res 91:672-80.

117. Hayward, G. S. 1999. KSHV strains: the origins and global spread of the virus. Semin Cancer Biol 9:187-99.

118. Hirano, T. 1991. Interleukin 6 (IL-6) and its receptor: their role in plasma cell neoplasias. Int J Cell Cloning 9:166-84.

119. Hitomi, M., and D. W. Stacey. 1999. Cyclin D1 production in cycling cells depends on ras in a cell-cycle-specific manner. Curr Biol 9:1075-84.

120. Ho, H. H., D. Du, and M. C. Gershengorn. 1999. The N terminus of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor is necessary for high affinity chemokine binding but not for constitutive activity. J Biol Chem 274:31327-32.

121. Ho, H. H., N. Ganeshalingam, A. Rosenhouse-Dantsker, R. Osman, and M. C. Gershengorn. 2001. Charged Residues at the Intracellular Boundary of Transmembrane Helices 2 and 3 Independently Affect Constitutive Activity of Kaposi's Sarcoma- associated Herpesvirus G Protein-coupled Receptor. J Biol Chem 276:1376-1382.

122. Huyer, G., and D. R. Alexander. 1999. Immune signalling: SHP-2 docks at multiple ports. Curr Biol 9:R129-32.

123. Imai, Y., M. Kurokawa, K. Izutsu, A. Hangaishi, K. Maki, S. Ogawa, S. Chiba, K. Mitani, and H. Hirai. 2001. Mutations of the Smad4 gene in acute myelogeneous leukemia and their functional implications in leukemogenesis. Oncogene 20:88-96.

124. Inman, G. J., F. J. Nicolas, and C. S. Hill. 2002. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. Mol Cell 10:283-94.

125. Isegawa, Y., Z. Ping, K. Nakano, N. Sugimoto, and K. Yamanishi. 1998. Human herpesvirus 6 open reading frame U12 encodes a functional beta-chemokine receptor. J Virol 72:6104-12.

126. Itoh, S., F. Itoh, M. J. Goumans, and P. Ten Dijke. 2000. Signaling of transforming growth factor-beta family members through Smad proteins. Eur J Biochem 267:6954-67.

127. Jones, K. D., Y. Aoki, Y. Chang, P. S. Moore, R. Yarchoan, and G. Tosato. 1999. Involvement of interleukin-10 (IL-10) and viral IL-6 in the spontaneous

growth of Kaposi's sarcoma herpesvirus-associated infected primary effusion lymphoma cells. Blood 94:2871-9.

128. Jordan, R., L. Schang, and P. A. Schaffer. 1999. Transactivation of herpes simplex virus type 1 immediate-early gene expression by virion-associated factors is blocked by an inhibitor of cyclin-dependent protein kinases. J Virol 73:8843-7.

129. Kaaya, E. E., C. Parravicini, C. Ordonez, R. Gendelman, E. Berti, R. C. Gallo, and P. Biberfeld. 1995. Heterogeneity of spindle cells in Kaposi's sarcoma: comparison of cells in lesions and in culture. J Acquir Immune Defic Syndr Hum Retrovirol 10:295-305.

130. Kadin, M. E., M. W. Cavaille-Coll, R. Gertz, J. Massague, S. Cheifetz, and D. George. 1994. Loss of receptors for transforming growth factor beta in human T-cell malignancies. Proc Natl Acad Sci U S A 91:6002-6.

131. Kedes, D. H., E. Operskalski, M. Busch, R. Kohn, J. Flood, and D. Ganem. 1996. The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nat Med 2:918-24.

132. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci. 1986. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 163:1037-50.

133. Keller, S. A., D. Hernandez-Hopkins, J. Vider, V. Ponomarev, E. Hyjek, E. J. Schattner, and E. Cesarman. 2006. NF-kappaB is essential for the

progression of KSHV- and EBV-infected lymphomas in vivo. Blood 107:3295-302.

134. Kiess, M., R. M. Gill, and P. A. Hamel. 1995. Expression and activity of the retinoblastoma protein (pRB)-family proteins, p107 and p130, during L6 myoblast differentiation. Cell Growth Differ 6:1287-98.

135. Kirshner, J. R., K. Staskus, A. Haase, M. Lagunoff, and D. Ganem. 1999. Expression of the open reading frame 74 (G-protein-coupled receptor) gene of Kaposi's sarcoma (KS)-associated herpesvirus: implications for KS pathogenesis. J Virol 73:6006-14.

136. Kivinen, L., M. Tsubari, T. Haapajarvi, M. B. Datto, X. F. Wang, and M. Laiho. 1999. Ras induces p21Cip1/Waf1 cyclin kinase inhibitor transcriptionally through Sp1-binding sites. Oncogene 18:6252-61.

137. Kledal, T. N., M. M. Rosenkilde, and T. W. Schwartz. 1998. Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. FEBS Lett 441:209-14.

138. Knaus, P. I., D. Lindemann, J. F. DeCoteau, R. Perlman, H. Yankelev, M. Hille, M. E. Kadin, and H. F. Lodish. 1996. A dominant inhibitory mutant of the type II transforming growth factor beta receptor in the malignant progression of a cutaneous T-cell lymphoma. Mol Cell Biol 16:3480-9.

139. Knowles, D. M., G. A. Chamulak, M. Subar, J. S. Burke, M. Dugan, J. Wernz, C. Slywotzky, G. Pelicci, R. Dalla-Favera, and B. Raphael. 1988. Lymphoid neoplasia associated with the acquired immunodeficiency syndrome (AIDS).

The New York University Medical Center experience with 105 patients (1981-1986). Ann Intern Med 108:744-53.

140. Knowles, D. M., G. Inghirami, A. Ubriaco, and R. Dalla-Favera. 1989. Molecular genetic analysis of three AIDS-associated neoplasms of uncertain lineage demonstrates their B-cell derivation and the possible pathogenetic role of the Epstein-Barr virus. Blood 73:792-799.

141. Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 252:668-74.

142. Kosugi, S., C. Van Dop, M. E. Geffner, W. Rabl, J. C. Carel, J. L. Chaussain, T. Mori, J. J. Merendino, Jr., and A. Shenker. 1995. Characterization of heterogeneous mutations causing constitutive activation of the luteinizing hormone receptor in familial male precocious puberty. Hum Mol Genet 4:183-8.

143. Kudoh, A., T. Daikoku, Y. Sugaya, H. Isomura, M. Fujita, T. Kiyono, Y. Nishiyama, and T. Tsurumi. 2004. Inhibition of S-phase cyclin-dependent kinase activity blocks expression of Epstein-Barr virus immediate-early and early genes, preventing viral lytic replication. J Virol 78:104-15.

144. Kudoh, A., M. Fujita, T. Kiyono, K. Kuzushima, Y. Sugaya, S. Izuta, Y. Nishiyama, and T. Tsurumi. 2003. Reactivation of lytic replication from B cells latently infected with Epstein-Barr virus occurs with high S-phase cyclin-dependent kinase activity while inhibiting cellular DNA replication. J Virol 77:851-61.

145. Kurokawa, M., K. Mitani, Y. Imai, S. Ogawa, Y. Yazaki, and H. Hirai. 1998. The t(3;21) fusion product, AML1/Evi-1, interacts with Smad3 and blocks transforming growth factor-beta-mediated growth inhibition of myeloid cells. Blood 92:4003-12.

146. Kurokawa, M., K. Mitani, K. Irie, T. Matsuyama, T. Takahashi, S. Chiba, Y. Yazaki, K. Matsumoto, and H. Hirai. 1998. The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3. Nature 394:92-6.

147. Lagunoff, M., J. Bechtel, E. Venetsanakos, A. M. Roy, N. Abbey, B. Herndier, M. McMahon, and D. Ganem. 2002. De Novo Infection and Serial Transmission of Kaposi's Sarcoma- Associated Herpesvirus in Cultured Endothelial Cells. J Virol 76:2440-8.

148. Laman, H., D. Coverley, T. Krude, R. Laskey, and N. Jones. 2001. Viral cyclin-cyclin-dependent kinase 6 complexes initiate nuclear DNA replication. Mol Cell Biol 21:624-35.

149. Landry, Y., and J. P. Gies. 2002. Heterotrimeric G proteins control diverse pathways of transmembrane signaling, a base for drug discovery. Mini Rev Med Chem 2:361-72.

150. Lebbe, C., L. Blum, C. Pellet, G. Blanchard, O. Verola, P. Morel, O. Danne, and F. Calvo. 1998. Clinical and biological impact of antiretroviral therapy with protease inhibitors on HIV-related Kaposi's sarcoma. Aids 12:F45-9.

151. Lee, H., R. Veazey, K. Williams, M. Li, J. Guo, F. Neipel, B. Fleckenstein, A. Lackner, R. C. Desrosiers, and J. U. Jung. 1998. Deregulation of cell growth

by the K1 gene of Kaposi's sarcoma-associated herpesvirus. Nat Med 4:435-40.

152. Lees, E. M., and E. Harlow. 1993. Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. Mol Cell Biol 13:1194-201.

153. Leger-Ravet, M. B., M. Peuchmaur, O. Devergne, J. Audouin, M. Raphael, J. Van Damme, P. Galanaud, J. Diebold, and D. Emilie. 1991. Interleukin-6 gene expression in Castleman's disease. Blood 78:2923-30.

154. Lennette, E. T., D. J. Blackbourn, and J. A. Levy. 1996. Antibodies to human herpesvirus type 8 in the general population and in Kaposi's sarcoma patients. Lancet 348:858-61.

155. Leurs, R., M. J. Smit, A. E. Alewijnse, and H. Timmerman. 1998. Agonist-independent regulation of constitutively active G-protein-coupled receptors. Trends Biochem Sci 23:418-22.

156. Li, M., H. Lee, J. Guo, F. Neipel, B. Fleckenstein, K. Ozato, and J. U. Jung. 1998. Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor. J Virol 72:5433-40.

157. Li, R. Y., F. Gaits, A. Ragab, J. M. Ragab-Thomas, and H. Chap. 1995. Tyrosine phosphorylation of an SH2-containing protein tyrosine phosphatase is coupled to platelet thrombin receptor via a pertussis toxin-sensitive heterotrimeric G-protein. Embo J 14:2519-26.

158. Liang, Y., and D. Ganem. 2004. RBP-J (CSL) is essential for activation of the K14/vGPCR promoter of Kaposi's sarcoma-associated herpesvirus by the lytic switch protein RTA. J Virol 78:6818-26.

159. Lin, S. F., R. Sun, L. Heston, L. Gradoville, D. Shedd, K. Haglund, M. Rigsby, and G. Miller. 1997. Identification, expression, and immunogenicity of Kaposi's sarcoma-associated herpesvirus-encoded small viral capsid antigen. J Virol 71:3069-76.

160. Linderoth, J., E. Rambech, and M. Dictor. 1999. Dominant human herpesvirus type 8 RNA transcripts in classical and AIDS-related Kaposi's sarcoma. J Pathol 187:582-7.

161. Liu, C., Y. Okruzhnov, H. Li, and J. Nicholas. 2001. Human herpesvirus 8 (HHV-8)-encoded cytokines induce expression of and autocrine signaling by vascular endothelial growth factor (VEGF) in HHV- 8-infected primary-effusion lymphoma cell lines and mediate VEGF- independent antiapoptotic effects. J Virol 75:10933-40.

162. Lorenz, U., K. S. Ravichandran, D. Pei, C. T. Walsh, S. J. Burakoff, and B. G. Neel. 1994. Lck-dependent tyrosyl phosphorylation of the phosphotyrosine phosphatase SH-PTP1 in murine T cells. Mol Cell Biol 14:1824-34.

163. MacGillivray, M., M. T. Herrera-Abreu, C. W. Chow, C. Shek, Q. Wang, E. Vachon, G. S. Feng, K. A. Siminovitch, C. A. McCulloch, and G. P. Downey. 2003. The protein tyrosine phosphatase SHP-2 regulates interleukin-1-induced ERK activation in fibroblasts. J Biol Chem 278:27190-8.

164. Macian, F., C. Lopez-Rodriguez, and A. Rao. 2001. Partners in transcription: NFAT and AP-1. Oncogene 20:2476-89.

165. Maeda, Y., U. Ikeda, M. Shimpo, S. Ueno, Y. Ogasawara, M. Urabe, A. Kume, T. Takizawa, T. Saito, P. Colosi, G. Kurtzman, K. Shimada, and K. Ozawa. 1998. Efficient gene transfer into cardiac myocytes using adeno-associated virus (AAV) vectors. J Mol Cell Cardiol 30:1341-8.

166. Margulies, B. J., H. Browne, and W. Gibson. 1996. Identification of the human cytomegalovirus G protein-coupled receptor homologue encoded by UL33 in infected cells and enveloped virus particles. Virology 225:111-25.

167. Markowitz, S., J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R. S. Fan, E. Zborowska, K. W. Kinzler, B. Vogelstein, and et al. 1995. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268:1336-8.

168. Martin, J. N., D. E. Ganem, D. H. Osmond, K. A. Page-Shafer, D. Macrae, and D. H. Kedes. 1998. Sexual transmission and the natural history of human herpesvirus 8 infection. N Engl J Med 338:948-54.

169. Masood, R., J. Cai, T. Zheng, D. L. Smith, Y. Naidu, and P. S. Gill. 1997. Vascular endothelial growth factor/vascular permeability factor is an autocrine growth factor for AIDS-Kaposi sarcoma. Proc Natl Acad Sci U S A 94:979-84.

170. Massague, J., L. Attisano, and J. L. Wrana. 1994. The TGF-beta family and its composite receptors. Trends Cell Biol 4:172-8.

171. Massague, J., and D. Wotton. 2000. Transcriptional control by the TGF-beta/Smad signaling system. Embo J 19:1745-54.

172. Mayol, X., J. Garriga, and X. Grana. 1996. G1 cyclin/CDK-independent phosphorylation and accumulation of p130 during the transition from G1 to G0 lead to its association with E2F-4. Oncogene 13:237-46.

173. Merritt, R., M. J. Hayman, and Y. M. Agazie. 2006. Mutation of Thr466 in SHP2 abolishes its phosphatase activity, but provides a new substrate-trapping mutant. Biochim Biophys Acta 1763:45-56.

174. Migone, T. S., N. A. Cacalano, N. Taylor, T. Yi, T. A. Waldmann, and J. A. Johnston. 1998. Recruitment of SH2-containing protein tyrosine phosphatase SHP-1 to the interleukin 2 receptor; loss of SHP-1 expression in human T-lymphotropic virus type I-transformed T cells. Proc Natl Acad Sci U S A 95:3845-50.

175. Milne, R. S., C. Mattick, L. Nicholson, P. Devaraj, A. Alcami, and U. A. Gompels. 2000. RANTES binding and down-regulation by a novel human herpesvirus-6 beta chemokine receptor. J Immunol 164:2396-404.

176. Min, J., and D. A. Katzenstein. 1999. Detection of Kaposi's sarcoma-associated herpesvirus in peripheral blood cells in human immunodeficiency virus infection: association with Kaposi's sarcoma, CD4 cell count, and HIV RNA levels. AIDS Res Hum Retroviruses 15:51-5.

177. Minoo, P., M. M. Zadeh, R. Rottapel, J. J. Lebrun, and S. Ali. 2004. A novel SHP-1/Grb2-dependent mechanism of negative regulation of cytokine-

receptor signaling: contribution of SHP-1 C-terminal tyrosines in cytokine signaling. Blood 103:1398-407.

178. Monini, P., S. Colombini, M. Sturzl, D. Goletti, A. Cafaro, C. Sgadari, S. Butto, M. Franco, P. Leone, S. Fais, P. Leone, G. Melucci-Vigo, C. Chiozzini, F. Carlini, G. Ascherl, E. Comali, C. Zietz, E. Ramazzotti, F. Ensoli, M. Andreoni, P. Pezzotti, G. Rezza, R. Yarchoan, R. C. Gallo, and B. Ensoli. 1999. Reactivation and persistence of human herpesvirus-8 infection in B cells and monocytes by Th-1 cytokines increased in Kaposi's sarcoma. Blood 93:4044-58.

179. Montaner, S., A. Sodhi, S. Pece, E. A. Mesri, and J. S. Gutkind. 2001. The Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor promotes endothelial cell survival through the activation of Akt/protein kinase B. Cancer Res 61:2641-8.

180. Moore, P. S., L. A. Kingsley, S. D. Holmberg, T. Spira, P. Gupta, D. R. Hoover, J. P. Parry, L. J. Conley, H. W. Jaffe, and Y. Chang. 1996. Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. Aids 10:175-80.

181. Morgan, D. O. 1997. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13:261-91.

182. Mori, N., M. Morishita, T. Tsukazaki, C. Z. Giam, A. Kumatori, Y. Tanaka, and N. Yamamoto. 2001. Human T-cell leukemia virus type I oncoprotein Tax represses Smad-dependent transforming growth factor beta signaling through interaction with CREB-binding protein/p300. Blood 97:2137-44.

183. Morris, C. B., R. Gendelman, A. J. Marrogi, M. Lu, J. M. Lockyer, W. Alperin-Lea, and B. Ensoli. 1996. Immunihistochemical detection of Bcl-2 in AIDS-associated and classical Kaposi's sarcoma. Am J Pathol 148:1055-63.

184. Motokura, T., T. Bloom, H. G. Kim, H. Juppner, J. V. Ruderman, H. M. Kronenberg, and A. Arnold. 1991. A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature 350:512-5.

185. Muralidhar, S., A. M. Pumfery, M. Hassani, M. R. Sadaie, N. Azumi, M. Kishishita, J. N. Brady, J. Doniger, P. Medveczky, and L. J. Rosenthal. 1998. Identification of kaposin (open reading frame K12) as a human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) transforming gene. J Virol 72:4980-8.

186. Nador, R. G., E. Cesarman, A. Chadburn, D. B. Dawson, M. Q. Ansari, J. Sald, and D. M. Knowles. 1996. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. Blood 88:645-56.

187. Nador, R. G., L. L. Milligan, O. Flore, X. Wang, L. Arvanitakis, D. M. Knowles, and E. Cesarman. 2001. Expression of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor monocistronic and bicistronic transcripts in primary effusion lymphomas. Virology 287:62-70.

188. Nakano, K., K. Tadagaki, Y. Isegawa, M. M. Aye, P. Zou, and K. Yamanishi. 2003. Human herpesvirus 7 open reading frame U12 encodes a functional beta-chemokine receptor. J Virol 77:8108-15.

189. Nakao, A., T. Imamura, S. Souchelnytskyi, M. Kawabata, A. Ishisaki, E. Oeda, K. Tamaki, J. Hanai, C. H. Heldin, K. Miyazono, and P. ten Dijke. 1997. TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. Embo J 16:5353-62.

190. Nandan, D., K. L. Knutson, R. Lo, and N. E. Reiner. 2000. Exploitation of host cell signaling machinery: activation of macrophage phosphotyrosine phosphatases as a novel mechanism of molecular microbial pathogenesis. J Leukoc Biol 67:464-70.

191. Nandan, D., T. Yi, M. Lopez, C. Lai, and N. E. Reiner. 2002. Leishmania EF-1alpha activates the Src homology 2 domain containing tyrosine phosphatase SHP-1 leading to macrophage deactivation. J Biol Chem 277:50190-7.

192. Neel, B. G., and N. K. Tonks. 1997. Protein tyrosine phosphatases in signal transduction. Curr Opin Cell Biol 9:193-204.

193. Neipel, F., J. C. Albrecht, and B. Fleckenstein. 1997. Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? J Virol 71:4187-92.

194. Neipel, F., J. C. Albrecht, and B. Fleckenstein. 1998. Human herpesvirus 8--the first human Rhadinovirus. J Natl Cancer Inst Monogr:73-7.

195. Neubig, R. R., M. Spedding, T. Kenakin, and A. Christopoulos. 2003. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. Pharmacol Rev 55:597-606.

196. Nicholas, J. 1996. Determination and analysis of the complete nucleotide sequence of human herpesvirus 7. J Virol 70:5975-89.

197. Nishi, J., and I. Maruyama. 2000. Increased expression of vascular endothelial growth factor (VEGF) in Castleman's disease: proposed pathomechanism of vascular proliferation in the affected lymph node. Leuk Lymphoma 38:387-94.

198. Noren-Muller, A., I. Reis-Correa, Jr., H. Prinz, C. Rosenbaum, K. Saxena, H. J. Schwalbe, D. Vestweber, G. Cagna, S. Schunk, O. Schwarz, H. Schiewe, and H. Waldmann. 2006. Discovery of protein phosphatase inhibitor classes by biology-oriented synthesis. Proc Natl Acad Sci U S A 103:10606-11.

199. Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol 15:2612-24.

200. Oka, T., T. Yoshino, K. Hayashi, N. Ohara, T. Nakanishi, Y. Yamaai, A. Hiraki, C. A. Sogawa, E. Kondo, N. Teramoto, K. Takahashi, J. Tsuchiyama, and T. Akagi. 2001. Reduction of hematopoietic cell-specific tyrosine phosphatase SHP-1 gene expression in natural killer cell lymphoma and various types of lymphomas/leukemias : combination analysis with cDNA expression array and tissue microarray. Am J Pathol 159:1495-505.

201. Olsen, S. J., Y. Chang, P. S. Moore, R. J. Biggar, and M. Melbye. 1998. Increasing Kaposi's sarcoma-associated herpesvirus seroprevalence with age in a highly Kaposi's sarcoma endemic region, Zambia in 1985. Aids 12:1921-5.

202. Ono, M., S. Bolland, P. Tempst, and J. V. Ravetch. 1996. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. Nature 383:263-6.

203. Oskenhendler, E., M. Duarte, and J. Soulier. 1996. Multicentric Castleman's disease in HIV infection: A clinical and pathological study of 20 patients. AIDS 10:61-67.

204. Osman, M., T. Kubo, J. Gill, F. Neipel, M. Becker, G. Smith, R. Weiss, B. Gazzard, C. Boshoff, and F. Gotch. 1999. Identification of human herpesvirus 8-specific cytotoxic T-cell responses. J Virol 73:6136-40.

205. Pagano, M., R. Pepperkok, J. Lukas, V. Baldin, W. Ansorge, J. Bartek, and G. Draetta. 1993. Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. J Cell Biol 121:101-11.

206. Parravinci, C., M. Corbellino, M. Paulli, U. Magrini, M. Lazzarino, P. S. Moore, and Y. Chang. 1997. Expression of a virus-derived cytokine, KSHV vIL-6, in HIV-seronegative Castleman's disease. Am J Pathol 151:1517-22.

207. Pasche, B., Y. Luo, P. H. Rao, S. D. Nimer, E. Dmitrovsky, P. Caron, L. Luzzatto, K. Offit, C. Cordon-Cardo, B. Renault, J. M. Satagopan, V. V. Murty, and J. Massague. 1998. Type I transforming growth factor beta receptor maps to 9q22 and exhibits a polymorphism and a rare variant within a polyalanine tract. Cancer Res 58:2727-32.

208. Pati, S., M. Cavrois, H. G. Guo, J. S. Foulke, Jr., J. Kim, R. A. Feldman, and M. Reitz. 2001. Activation of NF-kappaB by the human herpesvirus 8

chemokine receptor ORF74: evidence for a paracrine model of Kaposi's sarcoma pathogenesis. J Virol 75:8660-73.

209. Pati, S., J. S. Foulke, Jr., O. Barabitskaya, J. Kim, B. C. Nair, D. Hone, J. Smart, R. A. Feldman, and M. Reitz. 2003. Human herpesvirus 8-encoded vGPCR activates nuclear factor of activated T cells and collaborates with human immunodeficiency virus type 1 Tat. J Virol 77:5759-73.

210. Paulsen, S. J., M. M. Rosenkilde, J. Eugen-Olsen, and T. N. Kledal. 2005. Epstein-Barr virus-encoded BILF1 is a constitutively active G protein-coupled receptor. J Virol 79:536-46.

211. Peterson, B. A., and G. Frizzera. 1993. Multicentric Castleman's disease. Semin Oncol 20:636-47.

212. Platt, G., A. Carbone, and S. Mittnacht. 2002. p16INK4a loss and sensitivity in KSHV associated primary effusion lymphoma. Oncogene 21:1823-31.

213. Ponnazhagan, S., M. C. Yoder, and A. Srivastava. 1997. Adeno-associated virus type 2-mediated transduction of murine hematopoietic cells with long-term repopulating ability and sustained expression of a human globin gene in vivo. J Virol 71:3098-104.

214. Poole, A. W., and M. L. Jones. 2005. A SHPing tale: perspectives on the regulation of SHP-1 and SHP-2 tyrosine phosphatases by the C-terminal tail. Cell Signal 17:1323-32.

215. Premont, R. T., J. Inglese, and R. J. Lefkowitz. 1995. Protein kinases that phosphorylate activated G protein-coupled receptors. Faseb J 9:175-82.

216. Qu, C. K. 2000. The SHP-2 tyrosine phosphatase: signaling mechanisms and biological functions. Cell Res 10:279-88.

217. Qu, C. K., Z. Q. Shi, R. Shen, F. Y. Tsai, S. H. Orkin, and G. S. Feng. 1997. A deletion mutation in the SH2-N domain of Shp-2 severely suppresses hematopoietic cell development. Mol Cell Biol 17:5499-507.

218. Qu, C. K., W. M. Yu, B. Azzarelli, S. Cooper, H. E. Broxmeyer, and G. S. Feng. 1998. Biased suppression of hematopoiesis and multiple developmental defects in chimeric mice containing Shp-2 mutant cells. Mol Cell Biol 18:6075-82.

219. Rabkin, C. S., S. Janz, A. Lash, A. E. Coleman, E. Musaba, L. Liotta, R. J. Biggar, and Z. Zhuang. 1997. Monoclonal origin of multicentric Kaposi's sarcoma lesions. N Engl J Med 336:988-93.

220. Rao, V. R., G. B. Cohen, and D. D. Oprian. 1994. Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. Nature 367:639-42.

221. Regamey, N., M. Tamm, M. Wemli, A. Witschi, G. Thiel, G. Cathomas, and P. Erb. 1998. Transmission of human herpesvirus 8 infection from renal-transplant donors to recipients. N Engl J Med 339:1358-63.

222. Rezaee, S. A., C. Cunningham, A. J. Davison, and D. J. Blackbourn. 2006. Kaposi's sarcoma-associated herpesvirus immune modulation: an overview. J Gen Virol 87:1781-804.

223. Riedl, E., H. Strobl, O. Majdic, and W. Knapp. 1997. TGF-beta 1 promotes in vitro generation of dendritic cells by protecting progenitor cells from apoptosis. J Immunol 158:1591-7.

224. Rivard, N., F. R. McKenzie, J. M. Brondello, and J. Pouyssegur. 1995. The phosphotyrosine phosphatase PTP1D, but not PTP1C, is an essential mediator of fibroblast proliferation induced by tyrosine kinase and G protein-coupled receptors. J Biol Chem 270:11017-24.

225. Robinson, P. R., G. B. Cohen, E. A. Zhukovsky, and D. D. Oprian. 1992. Constitutively active mutants of rhodopsin. Neuron 9:719-25.

226. Roizman, B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf. 1981. Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses. Intervirology 16:201-17.

227. Roizman, B., and P. E. Pellett. 2001. p. 2381-2395. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology, 4th ed, vol. 2. Lippincott Williams and Wilkins, Philadelphia.

228. Roizmann, B., R. C. Desrosiers, B. Fleckenstein, C. Lopez, A. C. Minson, and M. J. Studdert. 1992. The family Herpesviridae: an update. Arch Virol 123:425-449.

229. Rosenkilde, M. M., and T. W. Schwartz. 2000. Potency of ligands correlates with affinity measured against agonist and inverse agonists but not against

neutral ligand in constitutively active chemokine receptor. Mol Pharmacol 57:602-9.

230. Ruscetti, F. W., S. Akel, and S. H. Bartelmez. 2005. Autocrine transforming growth factor-beta regulation of hematopoiesis: many outcomes that depend on the context. Oncogene 24:5751-63.

231. Russo, J., R. Bohenzky, M. Chien, J. Chen, M. Yan, D. Maddalena, J. Parry, D. Peruzzi, I. Edelman, Y. Chang, and P. Moore. 1996. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci U S A 93: p14862-7.

232. Salahuddin, S. Z., S. Nakamura, P. Biberfeld, M. H. Kaplan, P. D. Markham, L. Larsson, and R. C. Gallo. 1988. Angiogenic properties of Kaposi's sarcoma-derived cells after long-term culture in vitro. Science 242:430-3.

233. Samaniego, F., P. D. Markham, R. Gendelman, Y. Watanabe, V. Kao, K. Kowalski, J. A. Sonnabend, A. Pintus, R. C. Gallo, and B. Ensoli. 1998. Vascular endothelial growth factor and basic fibroblast growth factor present in Kaposi's sarcoma (KS) are induced by inflammatory cytokines and synergize to promote vascular permeability and KS lesion development. Am J Pathol 152:1433-43.

234. Sarid, R., O. Flore, R. A. Bohenzky, Y. Chang, and P. S. Moore. 1998. Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). J Virol 72:1005-12.

235. Sarid, R., S. Olsen, and P. Moore. 1999. Kaposi's sarcoma-associated herpesvirus: epidemiology, virology, and molecular biology. Advances in Virus Research 52:139-232.

236. Sarid, R., T. Sato, R. A. Bohenzky, J. J. Russo, and Y. Chang. 1997. Kaposi's sarcoma-associated herpesvirus encodes a functional bcl-2 homologue. Nat Med 3:293-8.

237. Sarid, R., J. S. Wiezorek, P. S. Moore, and Y. Chang. 1999. Characterization and cell cycle regulation of the major Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) latent genes and their promoter. J Virol 73:1438-46.

238. Saville, M. W., J. Lietzau, J. M. Pluda, I. Feuerstein, J. Odom, W. H. Wilson, R. W. Humphrey, E. Feigal, S. M. Steinberg, and S. Broder. 1995. Treatment of HIV-associated Kaposi's sarcoma with paclitaxel. Lancet 346:26-8.

239. Schiemann, W. P., W. M. Pfeifer, E. Levi, M. E. Kadin, and H. F. Lodish. 1999. A deletion in the gene for transforming growth factor beta type I receptor abolishes growth regulation by transforming growth factor beta in a cutaneous T-cell lymphoma. Blood 94:2854-61.

240. Schnabel, P., and M. Bohm. 1996. Heterotrimeric G proteins in heart disease. Cell Signal 8:413-23.

241. Schwarz, M., and P. M. Murphy. 2001. Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor constitutively activates NF-kappa B and induces proinflammatory cytokine and chemokine production via a C-terminal signaling determinant. J Immunol 167:505-13.

242. Seifert, R. 2004. The state of GPCR research in 2004. Nat Rev Drug Discov 3:575, 577-626.

243. Shenker, A., L. Laue, S. Kosugi, J. J. Merendino, Jr., T. Minegishi, and G. B. Cutler, Jr. 1993. A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. Nature 365:652-4.

244. Shepard, L. W., M. Yang, P. Xie, D. D. Browning, T. Voyno-Yasenetskaya, T. Kozasa, and R. D. Ye. 2001. Constitutive activation of NF-kappa B and secretion of interleukin-8 induced by the G protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus involve G alpha(13) and RhoA. J Biol Chem 276:45979-87.

245. Sherr, C. J. 2000. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res 60:3689-95.

246. Sherr, C. J., and J. M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13:1501-12.

247. Shultz, L. D. 1988. Pleiotropic effects of deleterious alleles at the "motheaten" locus. Curr Top Microbiol Immunol 137:216-22.

248. Shultz, L. D., P. A. Schweitzer, T. V. Rajan, T. Yi, J. N. Ihle, R. J. Matthews, M. L. Thomas, and D. R. Beier. 1993. Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. Cell 73:1445-54.

249. Sirianni, M. C., L. Vincenzi, V. Fiorelli, S. Topino, E. Scala, S. Uccini, A. Angeloni, A. Faggioni, D. Cerimele, F. Cottoni, F. Aiuti, and B. Ensoli. 1998. gamma-Interferon production in peripheral blood mononuclear cells and

tumor infiltrating lymphocytes from Kaposi's sarcoma patients: correlation with the presence of human herpesvirus-8 in peripheral blood mononuclear cells and lesional macrophages. Blood 91:968-76.

250. Smit, M. J., D. Verzijl, P. Casarosa, M. Navis, H. Timmerman, and R. Leurs. 2002. Kaposi's sarcoma-associated herpesvirus-encoded G protein-coupled receptor ORF74 constitutively activates p44/p42 MAPK and Akt via G(i) and phospholipase C-dependent signaling pathways. J Virol 76:1744-52.

251. Sodhi, A., S. Montaner, and J. S. Gutkind. 2004. Does dysregulated expression of a deregulated viral GPCR trigger Kaposi's sarcomagenesis? Faseb J 18:422-7.

252. Sodhi, A., S. Montaner, V. Patel, M. Zohar, C. Bais, E. A. Mesri, and J. S. Gutkind. 2000. The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. Cancer Res 60:4873-80.

253. Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M. F. d'Agay, J. P. Clauvel, M. Raphael, L. Degos, and et al. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood 86:1276-80.

254. Spiegel, A. M. 1996. Mutations in G proteins and G protein-coupled receptors in endocrine disease. J Clin Endocrinol Metab 81:2434-42.

255. Srikant, C. B., and S. H. Shen. 1996. Octapeptide somatostatin analog SMS 201-995 induces translocation of intracellular PTP1C to membranes in MCF-7 human breast adenocarcinoma cells. Endocrinology 137:3461-8.

256. Staskus, K. A., R. Sun, G. Miller, P. Racz, A. Jaslowski, C. Metroka, H. Brett-Smith, and A. T. Haase. 1999. Cellular tropism and viral interleukin-6 expression distinguish human herpesvirus 8 involvement in Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. J Virol 73:4181-7.

257. Staskus, K. A., W. Zhong, K. Gebhard, B. Hemdier, H. Wang, R. Renne, J. Beneke, J. Pudney, D. J. Anderson, D. Ganem, and A. T. Haase. 1997. Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol 71:715-9.

258. Streblow, D. N., C. Soderberg-Naucler, J. Vieira, P. Smith, E. Wakabayashi, F. Ruchti, K. Mattison, Y. Altschuler, and J. A. Nelson. 1999. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. Cell 99:511-20.

259. Streblow, D. N., J. Vomaske, P. Smith, R. Melnychuk, L. Hall, D. Pancheva, M. Smit, P. Casarosa, D. D. Schlaepfer, and J. A. Nelson. 2003. Human cytomegalovirus chemokine receptor US28-induced smooth muscle cell migration is mediated by focal adhesion kinase and Src. J Biol Chem 278:50456-65.

260. Strieter, R. M., P. J. Polverini, S. L. Kunkel, D. A. Arenberg, M. D. Burdick, J. Kasper, J. Dzuiba, J. Van Damme, A. Walz, D. Marriott, and et al. 1995. The

functional role of the ELR motif in CXC chemokine-mediated angiogenesis. J Biol Chem 270:27348-57.

261. Sturzl, M., H. Brandstetter, and W. K. Roth. 1992. Kaposi's sarcoma: a review of gene expression and ultrastructure of KS spindle cells in vivo. AIDS Res Hum Retroviruses 8:1753-63.

262. Sun, L., G. Wu, J. K. Willson, E. Zborowska, J. Yang, I. Rajkarunanayake, J. Wang, L. E. Gentry, X. F. Wang, and M. G. Brattain. 1994. Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. J Biol Chem 269:26449-55.

263. Sun, R., S. F. Lin, K. Staskus, L. Gradoville, E. Grogan, A. Haase, and G. Miller. 1999. Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. J Virol 73:2232-42.

264. Swanton, C., D. J. Mann, B. Fleckenstein, F. Neipel, G. Peters, and N. Jones. 1997. Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. Nature 390:184-7.

265. Symes, A., N. Stahl, S. A. Reeves, T. Farruggella, T. Servidei, T. Gearan, G. Yancopoulos, and J. S. Fink. 1997. The protein tyrosine phosphatase SHP-2 negatively regulates ciliary neurotrophic factor induction of gene expression. Curr Biol 7:697-700.

266. Tadagaki, K., K. Nakano, and K. Yamanishi. 2005. Human herpesvirus 7 open reading frames U12 and U51 encode functional beta-chemokine receptors. J Virol 79:7068-76.

267. Talbot, S. J., R. A. Weiss, P. Kellam, and C. Boshoff. 1999. Transcriptional analysis of human herpesvirus-8 open reading frames 71, 72, 73, K14, and 74 in a primary effusion lymphoma cell line. Virology 257:84-94.

268. Teruya-Feldstein, J., P. Zauber, J. E. Setsuda, E. L. Berman, L. Sorbara, M. Raffeld, G. Tosato, and E. S. Jaffe. 1998. Expression of human herpesvirus-8 oncogene and cytokine homologues in an HIV-seronegative patient with multicentric Castleman's disease and primary effusion lymphoma. Lab Invest 78:1637-42.

269. Thomas, J. A., L. A. Brookes, I. McGowan, I. Weller, and D. H. Crawford. 1996. HHV8 DNA in normal gastrointestinal mucosa from HIV seropositive people [letter]. Lancet 347:1337-8.

270. Todd, D. E., R. M. Densham, S. A. Molton, K. Balmanno, C. Newson, C. R. Weston, A. P. Garner, L. Scott, and S. J. Cook. 2004. ERK1/2 and p38 cooperate to induce a p21CIP1-dependent G1 cell cycle arrest. Oncogene 23:3284-95.

271. Tsutsumi, R., A. Takahashi, T. Azuma, H. Higashi, and M. Hatakeyama. 2006. Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA. Mol Cell Biol 26:261-76.

272. van den Heuvel, S., and E. Harlow. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. Science 262:2050-4.

273. Van Sande, J., J. Parma, M. Tonacchera, S. Swillens, J. Dumont, and G. Vassart. 1995. Somatic and germline mutations of the TSH receptor gene in thyroid diseases. J Clin Endocrinol Metab 80:2577-85.

274. Vassilatis, D. K., J. G. Hohmann, H. Zeng, F. Li, J. E. Ranchalis, M. T. Mortrud, A. Brown, S. S. Rodriguez, J. R. Weller, A. C. Wright, J. E. Bergmann, and G. A. Gaitanaris. 2003. The G protein-coupled receptor repertoires of human and mouse. Proc Natl Acad Sci U S A 100:4903-8.

275. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 24:179-89.

276. Veldhoen, M., and B. Stockinger. 2006. TGFbeta1, a "Jack of all trades": the link with pro-inflammatory IL-17-producing T cells. Trends Immunol 27:358-61.

277. Wakeling, M. N., D. J. Roy, A. A. Nash, and J. P. Stewart. 2001. Characterization of the murine gammaherpesvirus 68 ORF74 product: a novel oncogenic G protein-coupled receptor. J Gen Virol 82:1187-97.

278. Waldhoer, M., P. Casarosa, M. M. Rosenkilde, M. J. Smit, R. Leurs, J. L. Whistler, and T. W. Schwartz. 2003. The carboxyl terminus of human cytomegalovirus-encoded 7 transmembrane receptor US28 camouflages agonism by mediating constitutive endocytosis. J Biol Chem 278:19473-82.

279. Wang, H. W., M. W. Trotter, D. Lagos, D. Bourboulia, S. Henderson, T. Makinen, S. Elliman, A. M. Flanagan, K. Alitalo, and C. Boshoff. 2004. Kaposi sarcoma herpesvirus-induced cellular reprogramming contributes to the lymphatic endothelial gene expression in Kaposi sarcoma. Nat Genet 36:687-93.

280. Wang, T. H., H. S. Wang, and Y. K. Soong. 2000. Paclitaxel-induced cell death: where the cell cycle and apoptosis come together. Cancer 88:2619-28.

281. Whitby, D., M. R. Howard, M. Tenant-Flowers, N. S. Brink, A. Copas, C. Boshoff, T. Hatzioannou, F. E. Suggett, D. M. Aldam, and A. S. Denton. 1995. Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet 346:799-802.

282. Wiebusch, L., and C. Hagemeier. 1999. Human cytomegalovirus 86-kilodalton IE2 protein blocks cell cycle progression in G(1). J Virol 73:9274-83.

283. Williams, B. R. 1999. PKR; a sentinel kinase for cellular stress. Oncogene 18:6112-20.

284. Wolfraim, L. A., T. M. Fernandez, M. Mamura, W. L. Fuller, R. Kumar, D. E. Cole, S. Byfield, A. Felici, K. C. Flanders, T. M. Walz, A. B. Roberts, P. D. Aplan, F. M. Balis, and J. J. Letterio. 2004. Loss of Smad3 in acute T-cell lymphoblastic leukemia. N Engl J Med 351:552-9.

285. Wong, S. K. 2003. G protein selectivity is regulated by multiple intracellular regions of GPCRs. Neurosignals 12:1-12.

286. Wong, W. W. 1998. ICE family proteases in inflammation and apoptosis. Agents Actions Suppl 49:5-13.

287. Wrana, J. L., L. Attisano, R. Wieser, F. Ventura, and J. Massague. 1994. Mechanism of activation of the TGF-beta receptor. Nature 370:341-7.

288. Wu, D. Q., C. H. Lee, S. G. Rhee, and M. I. Simon. 1992. Activation of phospholipase C by the alpha subunits of the Gq and G11 proteins in transfected Cos-7 cells. J Biol Chem 267:1811-7.

289. Wu, X., J. H. Bayle, D. Olson, and A. J. Levine. 1993. The p53-mdm-2 autoregulatory feedback loop. Genes Dev 7:1126-32.

290. Xu, R., Y. Yu, S. Zheng, X. Zhao, Q. Dong, Z. He, Y. Liang, Q. Lu, Y. Fang, X. Gan, X. Xu, S. Zhang, X. Zhang, and G. S. Feng. 2005. Overexpression of Shp2 tyrosine phosphatase is implicated in leukemogenesis in adult human leukemia. Blood 106:3142-9.

291. Yang, T., S. Chen, M. Leach, D. Manfra, B. Homey, M. Wiekowski, L. Sullivan, C. Jenh, S. Narula, S. Chensue, and S. Lira. 2000. Transgenic expression of the chemokine receptor encoded by human herpesvirus 8 induces an angioproliferative disease resembling Kaposi's sarcoma. J Exp Med 191: p445-54.

292. Yen-Moore, A., S. D. Hudnall, P. L. Rady, R. F. Wagner, Jr., T. O. Moore, O. Memar, T. K. Hughes, and S. K. Tyring. 2000. Differential expression of the HHV-8 vGCR cellular homolog gene in AIDS-associated and classic Kaposi's sarcoma: potential role of HIV-1 Tat. Virology 267:247-51.

293. Yi, T., J. L. Cleveland, and J. N. Ihle. 1991. Identification of novel protein tyrosine phosphatases of hematopoietic cells by polymerase chain reaction amplification. Blood 78:2222-8.

294. Yi, T., A. L. Mui, G. Krystal, and J. N. Ihle. 1993. Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor beta chain and

down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis. Mol Cell Biol 13:7577-86.

295. You, M., D. H. Yu, and G. S. Feng. 1999. Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway. Mol Cell Biol 19:2416-24.

296. Yu, Y., J. B. Black, C. S. Goldsmith, P. J. Browning, K. Bhalla, and M. K. Offermann. 1999. Induction of human herpesvirus-8 DNA replication and transcription by butyrate and TPA in BCBL-1 cells. J Gen Virol 80:83-90.

297. Zeng, Y. X., and W. S. el-Deiry. 1996. Regulation of p21WAF1/CIP1 expression by p53-independent pathways. Oncogene 12:1557-64.

298. Zhang, Z., K. Shen, W. Lu, and P. A. Cole. 2003. The role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored via expressed protein ligation. J Biol Chem 278:4668-74.

299. Zhong, W., H. Wang, B. Herndier, and D. Ganem. 1996. Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. Proc Natl Acad Sci U S A 93:6641-6.

300. Ziegler, J. L., and E. Katongole-Mbidde. 1996. Kaposi's sarcoma in childhood: an analysis of 100 cases from Uganda and relationship to HIV infection. Int J Cancer 65:200-3.

301. Zoeteweij, J. P., A. V. Moses, A. S. Rinderknecht, D. A. Davis, W. W. Overwijk, R. Yarchoan, J. M. Orenstein, and A. Blauvelt. 2001. Targeted inhibition of calcineurin signaling blocks calcium-dependent reactivation of Kaposi sarcoma-associated herpesvirus. Blood 97:2374-80.

Trono. 1998. Self-inactivating lentivirus vector for safe and eff

gene delivery. J Virol 72:9873-80.